- Knorr, L. & Rössler, P. (1903). *Ber. dtsh. chem. Ges.* **36**, 1278.
- Luck, J. M. & Wilcox, A. (1953). *J. biol. Chem.* **205**, 859.
- Ohno, K. (1953). *J. Biochem., Tokyo*, **40**, 621.
- Rees, M. W. (1946). *Biochem. J.* **40**, 632.
- Sanger, F. (1945). *Biochem. J.* **39**, 507.
- Schlesinger, H. I. (1944). Quoted by J. R. Elliott, E. M. Boldebuck and G. F. Roedel (1952). *J. Amer. Chem. Soc.* **74**, 5049, footnote 11.
- Snyder, H. R. & Putnam, R. E. (1954). *J. Amer. Chem. Soc.* **76**, 1893.
- Thompson, A. R. (1952). *Nature, Lond.*, **169**, 495.
- Van Slyke, D. D. (1911). *J. biol. Chem.* **9**, 185.
- Waldkötter, K. F. (1938). *Rec. Trav. chim. Pays-Bas*, **57**, 1294.
- Walker, J. (1947). *J. chem. Soc.* p. 1552.
- Wittig, G. & Hornberger, P. (1952). *Liebigs Ann.* **577**, 11.
- Zahn, H. & Wurtz, A. (1952). *Biochem. Z.* **322**, 327.

Glycine Uptake by the Proteins of the Rat Pancreatic Juice

BY L. C. U. JUNQUEIRA, G. C. HIRSCH AND H. A. ROTHCHILD

Laboratory for Cell Physiology, Faculdade de Medicina, Universidade de São Paulo, Brasil

(Received 3 March 1955)

Although extensive research has been done on the morphology and cytophysiology of cell secretion (see reviews by Hirsch, 1931, 1939, 1955) few data exist on the chemical phenomena related to this process.

As a continuation of a series of studies on the biochemistry of cell secretion (Junqueira, 1951; Rabinovitch *et al.* 1952; Fernandes & Junqueira, 1953, 1955) experiments were performed in order to obtain data on the rate at which this process occurs.

The pancreas is a convenient organ to work with, for besides actively secreting proteins its activity can be stimulated or depressed by pharmacological agents. Furthermore, up to the present time no blood proteins have to our knowledge been proved to permeate this gland and to be excreted with pancreatic juice; if this in fact occurred, however, it would complicate the interpretation of the results obtained.

We present here the results obtained by studying the rate of appearance of radioactivity in the rat pancreatic-juice proteins after intravenous injections of radioactive glycine and radioactive plasma proteins. Furthermore, the protein-bound and 'non-protein' radioactivity of blood plasma was studied during the experiments.

MATERIAL AND METHODS

Adult male Wistar rats weighing from 250 to 380 g. (average 306 g.) were used throughout. They were starved 24 hr. previously but water was given *ad lib.* Anaesthesia was performed by injecting intraperitoneally 8.0 mg. of Numal (Roche) per 100 g. body weight.

Radioactivity in pancreatic juice after injection of glycine

In these experiments a total of nine rats were used. Pancreatic juice was collected in 10 μ l. glass pipettes from a polyethylene cannula introduced into the common biliary

and pancreatic duct 2-3 mm. above its entrance into the duodenal wall. To avoid contamination with bile a high ligature of the biliary duct was performed 5 mm. below its emergence from the liver. A continuous flow of pancreatic juice was promoted by intravenous injection into the femoral vein of 0.7 unit of secretin and 1.0 μ g. of carbamoylcholine (cerbachol) dissolved in 0.2 ml. saline per rat. This stimulus was repeated every hour up to the end of the experiment at half of the initial dose.

Approximately 5 min. after the first stimulus 3.3 μ g./100 g. body weight of [1-¹⁴C]glycine (specific activity, 32.5 μ g./mg., supplied by the Radiochemical Centre, Amersham) were injected at the rate of 1 ml./kg. body weight into the femoral vein.

Radioactivity assays. To assay the radioactivity of the pancreatic juice, 10 μ l. samples of pancreatic juice were periodically collected, mixed with 2 μ l. of 0.1 N-NaOH and spread out evenly on a stainless-steel planchet (3.11 sq.cm.) and immediately dried by heating at 40°. With this technique an average of fifteen samples of pancreatic juice and seven of plasma could be studied in each experiment. Radioactivity determinations were performed on a Tracerlab gas-flow counter. Every sample was counted three times and the counts agreed to within 3%. Samples were corrected for self-absorption by using a standard curve obtained from measurements of radioactive pancreatic-juice proteins.

Protein estimations. The amount of protein present in the samples was assayed by the method described by Lowry, Rosebrough, Farr & Randall (1951), previously checked by Kjeldahl nitrogen determinations. The average amount of protein in the samples was 261.9 μ g., with a range of 25.5-709.4.

Separation of protein-bound and non-protein radioactivity. In order to separate protein-bound radioactivity from radioactivity bound to smaller molecules (non-protein radioactivity) the following procedure was adopted. The planchets were immersed during 10 min. each in absolute ethanol, 0.5% collodion in ethanol-ether (1:1, by vol.), 70% (w/v) ethanol, 30% (w/v) ethanol and distilled water. They were then transferred to 10% (w/v) trichloroacetic acid for 3 hr. at 37° and afterwards immersed in distilled water for 12 hr. with five changes. Preliminary experiments showed that when radioactive glycine is added to non-radio-

active pancreatic juice and extracted as described, all the radioactivity is lost after only 2 hr. of immersion in water. The planchets were then washed with 35% and 70% (w/v) ethanol, absolute ethanol and six changes of ethanol-ether (1:1 by vol.) (10 min. each) in order to dissolve the collodion, and dried at 37°.

Radioactivity in pancreatic juice after injection of radioactive plasma proteins

Three rats were injected with radioactive plasma proteins prepared by ammonium sulphate precipitation of plasma obtained from oxalated blood of animals injected intravenously 6 hr. previously with 3.3 μ C glycine per 100 g. body weight. These proteins were redissolved in saline and had an average activity of 494 counts/mg. protein. Each animal received the equivalent of 4 ml. plasma.

The pancreatic juice was collected and assayed for radioactivity from animals stimulated by secretin and carbamoylcholine as described above.

Radioactivity of the plasma of animals injected with glycine

In six of the animals used for the pancreatic-juice experiments blood was collected hourly in oxalated tubes and 10 μ l. samples of plasma were assayed for free and protein-bound radioactivity as described for the pancreatic juice.

In three other animals blood was collected at short intervals soon after the glycine injection, in order to study the rate of disappearance of the non-protein radioactivity.

RESULTS

Incorporation of radioactivity into the pancreatic-juice proteins

The results obtained are presented in Table 1 and the curve of Fig. 1 represents the average result of 141 samples obtained from nine animals. It may be seen that the maximum radioactivity is attained 2½ hr. after injection of [¹⁴C]glycine. Appreciable radioactivity appears in the pancreatic-juice protein only 50 min. after the injection. Fig. 1 also shows the rate of pancreatic-juice production under our experimental conditions. Although these and other results related to the physiology of the rat pancreas are to be published later, this curve is presented here in order to stress the point that maximum radioactivity appears only almost 3 hr. after glycine injection, although the action of the first injection of secretin and carbamoylcholine on pancreatic secretion was quite prompt. It is interesting to observe the similarity of our curve to the one obtained by Askonas, Campbell & Work (1954) for the incorporation of methionine into goat-milk proteins.

Analysis of the radioactivity measurements of samples before and after ethanol, ether, trichloroacetic acid and water extractions showed no constant and significant difference, suggesting that practically all the radioactivity in the pancreatic juice was bound to proteins.

Table 1. *Radioactivity of rat pancreatic-juice proteins after injection of carbamoylcholine-secretin and [1-¹⁴C]glycine*

Results expressed in counts/min./mg. protein.

Time after glycine injection (min.)	Number of rats (n)	Average counts ($\bar{x}$)	Standard deviation (s)	Confidence interval*	
				95 %	$\bar{x} \pm t \frac{s}{\sqrt{n}}$
0-20	9	11	22	11 ± 17	
20-40	9	43	87	43 ± 67	
40-60	8	352	276	352 ± 231	
60-80	9	1841	1855	1841 ± 1426	
80-100	9	6356	7364	6356 ± 5660	
100-120	8	7861	6536	7861 ± 5481	
120-140	9	9990	6456	9990 ± 4962	
140-160	9	9407	4699	9407 ± 3612	
160-180	9	11486	5960	11486 ± 4225	
180-200	9	10791	3261	10791 ± 2507	
200-220	9	10439	5747	10439 ± 4417	
220-240	8	9387	2675	9387 ± 2235	
240-260	9	8203	2144	8203 ± 1648	
260-280	6	9198	2866	9198 ± 3007	
280-300	5	6934	3355	6934 ± 4158	
300-320	5	7438	2489	7438 ± 3085	
320-340	4	5096	843	5096 ± 1341	
340-360	2	4507	1518	4507 ± 13641	
360-380	3	5001	842	5001 ± 2092	
380-400	2	4759	1389	4759 ± 12481	
400-420	2	3300	1131	3300 ± 10163	
420-440	2	2814	733	2814 ± 6587	

* t = the critical value of the statistic ' t ' (Student's table) at the 5% level with $n - 1$ degrees of freedom.

A possible source of error in the evaluation of the rate of appearance of radioactivity in the proteins of the pancreatic juice might be due to the time taken by these proteins to get from the cells to the distal end of the pancreatic duct. Inorganic phosphate is a normal component of pancreatic juice (La Barre, 1953) and the rate of its appearance might indicate the time taken by the juice to pass

Fig. 1. Appearance of radioactivity in the rat pancreatic-juice proteins after injection of $3.3 \mu\text{C}$ $[1-^{14}\text{C}]$ glycine and after secretin-carbamoylcholine stimulation. Δ , each point is the average radioactivity of samples from nine animals; $\circ$, rate of pancreatic juice flow. The asterisk indicates the time of the first stimulation and the arrow the time at which glycine was injected.

through the pancreatic duct. Sodium $[^{32}\text{P}]$ phosphate ($60 \mu\text{C}$, supplied by Abbott Laboratories) was therefore injected into three rats and the pancreatic juice collected and assayed for radioactivity as described above. In all rats injected with sodium $[^{32}\text{P}]$ phosphate a distinct radioactivity (300–400 counts/min.) could be observed 3 min. after the injection; the time after which the peak of ^{32}P radioactivity was reached was approximately 1 hr. These animals were studied for $2\frac{1}{2}$ hr. and a gradual decrease of this radioactivity was observed.

This result suggests that no appreciable time is lost by the juice proteins to pass from the pancreatic cells to the main excretory duct. This point of view is corroborated by the results of Ball, Tucker, Solomon & Vennesland (1941), who described the prompt appearance of intravenously injected radioactive bicarbonate in pancreatic juice.

Radioactivity of pancreatic juice after injection of radioactive plasma proteins

In the three rats studied no radioactivity above background was observed in twenty-two samples of pancreatic juice collected during 6 hr.

Radioactivity in plasma of animals injected with $[^{14}\text{C}]$ glycine

The appearance of radioactivity in the plasma after glycine injection is shown in Fig. 2. The protein-bound radioactivity reaches a maximum after 4 hr. The non-protein radioactivity, however, decreases rapidly, reaching a very low level after 1 hr. and disappearing after 8 hr.

Fig. 2. Appearance of radioactivity in the blood plasma proteins of the rat after injection of $[^{14}\text{C}]$ glycine. Δ , Each point is the average radioactivity of samples from six rats; $\circ$, non-protein plasma, each point is the average radioactivity from six rats.

DISCUSSION

The protein-bound radioactivity in pancreatic juice reaches its maximum about 3 hr. after the injection of $[1-^{14}\text{C}]$ glycine (Fig. 1) although the first injection of secretin and carbamoylcholine stimulates maximum flow of juice much earlier. The slope of the radioactivity curve for the juice proteins gives an idea of the rate of pancreatic secretion and consequently the time necessary for the synthesis of the proteins of the pancreatic juice. It is of interest to compare these results with those obtained by other authors who studied pancreas secretion even though different methods and species were used. Hirsch (1932) described a complete restitution of secretory granules in mouse pancreas, 12–14 hr. after pilocarpine administration. Secretory granules, however, appeared after about 3 hr. van Weel & Engel (1938), Daly & Mirsky (1953) and Sesso (personal communication) obtained in pilocarpine-stimulated mice resynthesis of carboxypeptidase and amylase activity respectively 12 and 7 hr. after the injection. Mathematical calculations of Langstroth, McRae & Komarov (1939), based on the rate of protein secretion by the dog pancreas, indicate that the

time necessary for the restoration of secretory granules from a previously depleted gland would be about 3 hr.

Our results on the incorporation of glycine into plasma proteins agree with those of Friedberg, Tarver & Greenberg (1948), and the curve of disappearance of non-protein radioactivity from blood plasma presented by us agrees with the data obtained by Miller, Bly & Bale (1954).

The fact that no radioactivity was detected in the pancreatic juice of rats injected with radioactive plasma proteins shows that these proteins do not contribute appreciably to the formation of pancreatic-juice proteins. This conclusion is supported by the observation that after injection of labelled glycine the peak in the radioactivity of the pancreatic-juice proteins occurred before that of the plasma proteins. If plasma proteins were utilized for the synthesis of pancreatic-juice proteins, the peak in the radioactivity of the latter would be expected to occur some time after the plasma proteins had reached maximum radioactivity and would be less than that of the plasma proteins (Zilversmit, Entenman & Fishler, 1943; Hevesy, 1948).

Although direct utilization of plasma proteins for milk-protein synthesis has been suggested by Graham, Peterson, Houchin & Turner (1937), more recent observations by Askonas *et al.* (1954) do not support this view, and the present work suggests that pancreatic-juice proteins are also not synthesized directly from plasma proteins.

The almost complete absence of radioactivity in the pancreatic-juice proteins in the first 50 min. after injection of glycine suggests that there is no appreciable interchange between free glycine and protein-bound glycine, since this would lead to an earlier appearance of radioactivity in the pancreatic-juice proteins.

The present observations thus suggest that the proteins of rat pancreatic juice are derived mainly from free amino acids. Recently Askonas *et al.* (1954) came to a similar conclusion about the formation of goat-milk proteins.

SUMMARY

1. Rats were injected with [^{14}C]glycine or with radioactive plasma proteins, and the appearance of radioactivity in the pancreatic juice proteins was followed.

2. Maximum radioactivity was observed $2\frac{1}{2}$ hr. after the injection of [^{14}C]glycine.

3. No appreciable radioactivity was observed in the first 50 min. after the glycine injection.

4. The appearance of protein-bound and non-protein radioactivity in the plasma of animals injected with glycine was also followed.

5. No radioactivity could be detected in the pancreatic juice of animals injected with radioactive plasma proteins.

The significance of these results is discussed.

One of the authors (G. C. Hirsch of the Department of Zoology, University of Göttingen) is grateful to the Forschungsgemeinschaft and the University of São Paulo who made his tenure as visiting professor possible. The authors are grateful to the Isotope Laboratory of the University of São Paulo for constant help and to the Conselho Nacional de Pesquisas for funds. The technical assistance of Miss M. C. Rosa and Miss O. Castellani is acknowledged.

REFERENCES

- Askonas, B. A., Campbell, P. N. & Work, T. S. (1954). *Biochem. J.* **58**, 326.
 Ball, E. G., Tucker, H. F., Solomon, A. K. & Vennesland, B. (1941). *J. biol. Chem.* **140**, 119.
 Daly, M. M. & Mirsky, A. E. (1953). *J. gen. Physiol.* **36**, 243.
 Fernandes, J. F. & Junqueira, L. C. U. (1953). *Exp. Cell Res.* **5**, 329.
 Fernandes, J. F. & Junqueira, L. C. U. (1955). *Arch. Biochem. Biophys.* (in the Press).
 Friedberg, F., Tarver, H. & Greenberg, D. M. (1948). *J. biol. Chem.* **173**, 355.
 Graham, W. R. jun., Peterson, V. E., Houchin, O. B. & Turner, C. W. (1937). *J. biol. Chem.* **122**, 275.
 Hevesy, G. (1948). *Radioactive Indicators*. New York: Interscience Publ. Inc.
 Hirsch, G. C. (1931). *Biol. Rev.* **6**, 88.
 Hirsch, G. C. (1932). *Z. Zellforsch.* **15**, 290.
 Hirsch, G. C. (1939). *Form- und Stoffwechsel der Golgi-Körper*. Berlin: Borntraeger.
 Hirsch, G. C. (1955). *Stoffwechselbiologie des Cytoplasmas*. Handb. d. Allg. Pathologie, Vol. 2. Springer-Verlag.
 Junqueira, L. C. U. (1951). *Exp. Cell Res.* **2**, 327.
 La Barre, J. (1953). *Tabul. biol. Hague*, **21**, 30.
 Langstroth, G. O., McRae, D. R. & Komarov, S. A. (1939). *Canad. J. Res. D*, **17**, 137.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
 Miller, L. L., Bly, C. G. & Bale, W. F. (1954). *J. exp. Med.* **99**, 133.
 Rabinovitch, M., Valeri, V., Rothschild, H. A., Camara, S., Sesso, A. & Junqueira, L. C. U. (1952). *J. biol. Chem.* **198**, 815.
 Weel, P. B. van & Engel, C. (1938). *Acta brev. neerl. Physiol.* **8**, 156.
 Zilversmit, D. B., Entenman, C. & Fishler, M. C. (1943). *J. gen. Physiol.* **26**, 325.