Biochemical Pharmacology, Vol. 19, pp. 853–857. Pergamon Press. 1970. Printed in Great Britain

# EFFECT OF INDOSPICINE ON INCORPORATION OF L-ARGININE-$^{14}$C INTO PROTEIN AND TRANSFER RIBONUCLEIC ACID BY CELL-FREE SYSTEMS FROM RAT LIVER

N. P. MADSEN, G. S. CHRISTIE and M. P. HEGARTY

Department of Pathology, University of Melbourne, Parkville, Victoria 3052, and C.S.I.R.O. Division of Tropical Pastures, Cunningham Laboratory, St. Lucia, Queensland 4067, Australia

(*Received* 15 *July* 1969; *accepted* 15 *September* 1969)

**Abstract**—Incorporation of L-arginine-$^{14}$C (U) into protein was depressed in cell-free fractions from the livers of female rats treated with indospicine. The inhibitory factor appeared to be located in the 100,000 *g* supernatant. Depression of arginine incorporation was also observed on addition of indospicine *in vitro*. Raising the level of arginine in the reaction mixtures decreased the magnitude of the effect. The formation of $^{14}$C-arginyl-*t*RNA was inhibited by indospicine *in vitro*. Under similar conditions, formation of $^{14}$C-leucyl-*t*RNA was not impaired. Possible mechanisms relating to the antagonism between indospicine and arginine are discussed.

IN A PREVIOUS communication it was reported that inhibition of incorporation of leucine-1-$^{14}$C into liver and serum protein occurred *in vivo* after administration of indospicine (L-2-amino-6-amidinohexanoic acid) to rats.[1] This paper reports studies designed to elucidate the mechanism of the inhibition. The effect of *in vivo* administration of indospicine, and of its presence *in vitro*, on $^{14}$C-arginine incorporation by cell-free reaction systems was investigated because of the close structural relationship between arginine and indospicine.

## METHODS

Female Sprague–Dawley rats (170–220 g) were used. They were fed a complete pellet diet but were starved overnight before killing. In experiments involving treatment of the whole animal the rats received 2 g/kg indospicine as a 20% (w/v) aqueous solution by stomach tube, and the controls received a corresponding volume of water (1 ml/100 g body weight). The animals were killed 12 hr after treatment. At this time interval $^{14}$C-amino acid incorporation had been previously shown to be inhibited *in vivo*.[1]

The liver microsomal and 100,000 *g* supernatant fractions used for assaying incorporation into protein in a cell-free system were prepared as described by Weksler and Gelboin.[2] The assay mixtures used were similar to those described by these authors except for the modifications shown in Tables 1 and 2.

For the experiment in which the $^{14}$C-aminoacylation of transfer RNA (*t*RNA) was investigated, a crude mixture of amino acid-activating enzymes was prepared according to the method of Axel *et al.*,[3] whose incorporation system was also used (Table 3).

$^{14}$C-protein radioactivity was measured using a toluene–Triton X-100 liquid

TABLE 1. EFFECT OF INDOSPICINE ADMINISTRATION TO RATS ON INCORPORATION OF L-ARGININE-$^{14}$C (U) INTO PROTEIN *IN VITRO* AND SUBCELLULAR LOCALIZATION OF THE INHIBITORY FACTOR*

| Source | | cpm/mg microsomal protein† |
|---|---|---|
| Microsomes | Supernatant | |
| Control | Control | 354 (322–389) |
| Treated | Treated | 222 (184–260) |
| Control | Treated | 303 (259–318) |
| Treated | Control | 366 (334–398) |

* Reaction mixtures, prepared in duplicate, contained in a final volume of 2 ml: potassium phosphate buffer, pH 7·4, 12 mM; ATP, 1·5 mM; GTP, 0·3 mM; $MgCl_2$, 12 mM; phosphoenolpyruvate, 15 mM; pyruvate kinase, 0·15 mg; 2-mercaptoethanol, 6 mM; sucrose, 77 mM; L-arginine-$^{14}$C(U)HCl, 0·094 mM (1 $\mu$c); 0·2 ml of 100,000 *g* supernatant (approx. 3 mg protein); and 0·4 ml of microsomal suspension (5–6 mg protein). After incubation at 37° for 15 min the reaction was stopped by the addition of 2 ml of 20% trichloroacetic acid. The precipitated protein was washed and prepared for liquid scintillation counting of radioactivity as described under Methods.

† The data represent mean values of three experiments, each carried out in duplicate, with the range of results shown in parentheses.

TABLE 2. INHIBITION OF INCORPORATION OF L-ARGININE-$^{14}$C (U) INTO PROTEIN BY ADDITION OF INDOSPICINE *IN VITRO* AND ANTAGONISM TOWARDS THE EFFECT BY INCREASED ARGININE*

| Conc. indospicine present (mM) | Conc. arginine added (mM) | cpm/mg microsomal protein | Inhibition (%) |
|---|---|---|---|
| — | 0·071 | 479 | |
| 1·1 | 0·071 | 266 | 44 |
| — | 2·83 | 131 | |
| 1·1 | 2·83 | 128 | 2 |
| — | 0·094 | 499 | |
| 4·4 | 0·094 | 171 | 66 |
| — | 2·83 | 149 | |
| 4·4 | 2·83 | 118 | 21 |
| — | 0·094 | 322 | |
| 8·8 | 0·094 | 54 | 83 |
| — | 2·83 | 122 | |
| 8·8 | 2·83 | 81 | 34 |

* Assay procedures were as described in Table 1 except that when the amount of arginine was increased by also including L-arginine-$^{12}$C, 5 $\mu$c of L-arginine-$^{14}$C(U) were used to partially offset the diluting effect of the addition on $^{14}$C-arginine availability. Concentrations shown are the sum of $^{14}$C- and $^{12}$C-arginine added.

scintillation mixture,[4] subsequent to purification of the samples,[5] and solubilization in 1N NaOH. $^{14}$C-aminoacyl-*t*RNA radioactivity of samples on filter paper discs was determined by liquid scintillation counting after washing the discs according to the procedure of Nishimura and Novelli.[6]

Protein content of the microsomal suspensions was estimated by a biuret method.[7] Clarification of the samples was achieved by shaking with 3 ml $CHCl_3$ followed by

centrifugation. Microsomal RNA content was determined on 0·1 ml aliquots of the suspensions by the procedure described by Blobel and Potter.[8]

The following substances were purchased from Sigma Chemical Company: ATP (disodium salt), GTP (sodium salt), phosphoenolpyruvate (trisodium salt), pyruvate kinase, and soluble RNA (calf liver). The $^{14}$C-amino acids used were supplied by The Radiochemical Centre, Amersham. The specific activities were: L-arginine-$^{14}$C (U) HCl, 5·3 mc/m-mole (see Tables 1 and 2) and 10·7 mc/m-mole (Table 3); DL-leucine-1-$^{14}$C, 34 mc/m-mole.

TABLE 3. EFFECT OF INDOSPICINE *IN VITRO* ON THE CHARGING OF *t*RNA WITH L-ARGININE-$^{14}$C (U) OR DL-LEUCINE-1-$^{14}$C*

| Sample | Indospicine (mM) | Activity (cpm/sample) | % of control value |
|---|---|---|---|
| L-arginine-$^{14}$C (U) | none | 780 | 100 |
| | 0·1 | 780 | 100 |
| | 1·0 | 655 | 84 |
| | 2·5 | 380 | 49 |
| | 5·0 | 218 | 28 |
| | 10·0 | 0 | 0 |
| DL-leucine-1-$^{14}$C | none | 2640 | 100 |
| | 10·0 | 2680 | 101 |

* Reaction mixtures contained in a final volume of 1 ml: Tris-HCl buffer, pH 7·2, 0·1 M; $MgCl_2$, 5 mM; ATP, pH 7·2, 10 mM; L-arginine-$^{14}$C (U) HCl, 0·094 mM, or DL-leucine-1-$^{14}$C, 0·029 mM, (1 $\mu$c in each case); 1 mg calf liver tRNA; and enzyme fraction, 5·4 mg protein (approximately 6·5 $E_{260}$ units). Incubation was carried out for 10 min at 37°. The reaction was stopped by transferring duplicate 100 $\mu$l aliquots onto filter paper discs with a Marburg pipette, briefly drying in a stream of warm air, and finally depositing the discs in a beaker containing cold 10% trichloroacetic acid plus 10 mM $^{12}$C-amino acid. After further washing (see under Methods) radioactivity was measured in a liquid scintillation spectrometer. The data shown above represent values obtained after subtraction of the activity of zero-time samples without or with the appropriate amounts of indospicine.

## RESULTS

A preliminary experiment was performed to determine the effect of indospicine treatment of the whole animal on incorporation of $^{14}$C-arginine into protein in a cell-free system prepared from the liver. Comparative incorporation was 223 and 422 cpm/mg microsomal protein using fractions from treated and control rats respectively, these values being means of duplicate samples. The experiment was repeated with the additional procedure of cross-combining the microsomal and supernatant fractions of the control and treated groups. It was thus confirmed that incorporation was impaired in the presence of the fractions from a treated animal (Table 1). Furthermore, while the addition of control supernatant to treated microsomes restored incorporation to control levels, the addition of treated supernatant to control microsomes depressed incorporation.

These findings suggested that a factor in the supernatant fraction of the treated rats was depressing incorporation of $^{14}$C-arginine into protein in the cell-free system. The microsomal RNA/protein ratio was unaltered by the treatment (mean values for

three determinations being 0·132 both for control and treated groups), thus supporting the conclusion that the microsomal fraction was not the site of the inhibition. Since it was feasible that indospicine itself, or a metabolite, was the factor involved, the action of various concentrations of indospicine on the incorporating system was studied *in vitro*, using liver fractions isolated from untreated animals. To examine the possibility of antagonism between arginine and indospicine, in each set of experiments additional control and test reaction mixtures were prepared in each of which 1 mg of non-radioactive arginine was included.

It was found that, in the presence of indospicine, $^{14}$C-arginine incorporation into protein in the cell-free system was decreased (Table 2). The effect of indospicine was shown to be concentration-dependent though not in a linear manner. When the amount of arginine in the reaction mixtures was raised, the extent of the inhibition of incorporation due to indospicine was greatly reduced. The results indicated that indospicine itself may have been the agent present in the supernatant fraction from treated rats responsible for inhibited $^{14}$C-arginine incorporation, and that there appeared to be a competitive antagonism between the two amino acids with respect to some key factor in the incorporation system.

Since the first step in polypeptide synthesis involves activation of the amino acids and their subsequent attachment to *t*RNA, the effect of indospicine *in vitro* on the formation of $^{14}$C-arginyl-*t*RNA was determined. A progressive decrease in incorporation was observed in the presence of from 1·0 to 10 mM indospicine when $^{14}$C-arginyl-*t*RNA formation was completely abolished (Table 3). In contrast, $^{14}$C-leucyl-*t*RNA formation was not impaired in the presence of 10 mM indospicine.

## DISCUSSION

The findings presented here strongly suggest that competitive antagonism occurs between arginine and indospicine such that the latter can prevent arginine being incorporated into *t*RNA as a prelude to ribosomal polypeptide synthesis. The action of indospicine may possibly be mediated by inhibitory binding to either arginyl-*t*RNA synthetase or to arginine *t*RNA. It has been reported that proflavine[9] and steffimycin[10] bind strongly to *t*RNA, and the authors concluded that the binding was responsible for the interference with protein synthesis which they demonstrated in cell-free systems. On the other hand, Yellin[11] has suggested that the inhibitory effect of gamma aminobutyramide on incorporation of $^{14}$C-leucine into protein in Ehrlich ascites cell suspensions was due to competitive inhibition of the binding of glutamine to glutaminyl-*t*RNA synthetase. In support, the author cited evidence of the ability of glutamine to relieve the inhibition by gamma aminobutyramide. In addition, the activation of $^{14}$C-glutamine but not of $^{14}$C-leucine by an appropriate enzyme system from *E. coli* was inhibited by gamma aminobutyramide. Thus, because of the close analogy between the findings with indospicine and those with gamma aminobutyramide it is tentatively concluded that indospicine exerts its effects by inhibition of the binding of arginine to arginyl-*t*RNA synthetase. The recent publication of a method for the purification of rat liver arginyl-*t*RNA synthetase[12] will permit this postulate to be tested. Also, should the postulate prove to be correct the availability of the purified enzyme will enable the stoichiometric relationships of arginine and indospicine to be more precisely defined than has thus far been possible

because of the relatively crude nature of the cell-free preparations which were employed.

Though in indospicine-treated rats the incorporation of leucine-1-$^{14}$C into liver and serum protein was shown to be impaired,[1] the specific inhibitory effect of indospicine on the formation of $^{14}$C-arginyl-*t*RNA rather than on the formation of $^{14}$C-leucyl-*t*RNA, as shown in the cell-free system, would indicate that the effect of indospicine treatment on leucine incorporation *in vivo* was secondary to a primary interference with arginine incorporation. Preliminary experiments have shown that, in contrast to the case of arginine, the ability of liver microsomal-supernatant fractions from indospicine-treated rats to incorporate $^{14}$C-leucine, -lysine, or -phenylalanine into protein was not markedly impaired in the cell-free system.

*Acknowledgement*—This work was made possible by financial support from the Wellcome Trust and the Trustees of the Sir A. E. Rowden White Trust.

## REFERENCES

1. G. S. Christie, N. P. Madsen and M. P. Hegarty, *Biochem. Pharmac.* **18**, 693 (1969).
2. M. E. Weksler and H. V. Gelboin, *Biochim. biophys. Acta* **145**, 184 (1967).
3. R. Axel, I. B. Weinstein and E. Farber, *Proc. nat. Acad. Sci. U.S.A.* **58**, 1255 (1967).
4. N. P. Madsen, *Anal. Biochem.* **29**, 542 (1969).
5. C. M. Radding and D. Steinberg, *J. clin. Invest.* **39**, 1560 (1960).
6. S. Nishimura and G. D. Novelli, *Biochim. biophys. Acta* **80**, 574 (1964).
7. A. G. Gornall, C. J. Bardawill and M. M. David, *J. biol. Chem.* **177**, 751 (1949).
8. G. Blobel and V. R. Potter, *Biochim. biophys. Acta* **166**, 48 (1968).
9. T. Finkelstein and I. B. Weinstein, *J. biol. Chem.* **242**, 3763 (1967).
10. F. Reusser, *Biochem. Pharmac.* **18**, 287 (1969).
11. T. O. Yellin, *Biochem. biophys. Res. Commun.* **32**, 307 (1968).
12. H. Ikegami and A. C. Griffin, *Biochim. biophys. Acta* **178**, 166 (1969).