Incorporation of L-[1-¹⁴C]Leucine into Protein by Liver Postmitochondrial Supernatant: Opposing Effects of Preincubated Nicotinamide–Adenine Dinucleotide Phosphate and 4-Dimethylamino-3'-methylazobenzene

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Combination of a preincubated drug-metabolizing medium containing NADP⁺ with a cell-free protein-synthesizing system resulted in marked stimulation of incorporation of L-[1-¹⁴C]leucine into protein. Addition of 4-dimethylamino-3'-methylazobenzene, present and previously preincubated in the drug-metabolizing medium, decreased this effect.

Considerable work remains to be done to understand the initial interactions of chemical carcinogens with cellular processes. To this end the effects *in vitro* of the hepatocarcinogen 4-dimethylamino-3'-methylazobenzene on cell-free protein synthesis are being studied. The ¹⁴C-labelled amino acid-incorporating system chosen was that using liver postmitochondrial supernatant fractions essentially as described by Richardson *et al.* (1971). It has the advantage over more highly fractionated systems of being more active, and of being capable of detecting changes in the initiation phase of polypeptide formation (Pain, 1973).

It is commonly agreed that many carcinogens including, 4-dimethylamino-3'-methylazobenzene, require metabolic transformation in order to act (Miller, 1970). Accordingly this substance was exposed to a mixture supplemented with cofactors which promote biotransformation (Fouts, 1971) and which also included postmitochondrial supernatant as a source of microsomal drug-metabolizing enzymes. After this preincubation, the samples were combined with a second mixture to form the above-mentioned protein-synthesizing system. It was found that the presence of added nicotinamide nucleotides markedly stimulated amino acid incorporation, an effect antagonized by the concurrent presence of 4-dimethylamino-3'-methylazobenzene in the preincubation media.

Materials and methods

Materials. ATP, Hepes [2-(N-2-hydroxyethyl)piperazin-N'-yl]ethanesulphonic acid], NADP⁺, pyruvate kinase and phosphoenolpyruvate (trisodium salt) were products of Sigma Chemical Co., St. Louis, Mo., U.S.A. Glucose 6-phosphate and NAD⁺ were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. 4-Dimethylamino-3'-methylazobenzene (m.p. 119°C) was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. L-[1-¹⁴C]Leucine (56mCi/mmol) was supplied by The

Radiochemical Centre, Amersham, Bucks., U.K. Compound SKF 525A (2-diethylaminoethyl 2,2-diphenylvalerate hydrochloride) was a gift from Smith, Kline and French Laboratories, Philadelphia, Pa., U.S.A. All other reagents were of analytical grade.

Animals. Male Sprague–Dawley rats (200–250g) from the colony maintained in this Institution were used. The normal diet of rat pellets and water was available *ad libitum* until killing. Starvation was avoided because of its depressant effect on amino acid-incorporation rates (Richardson *et al.*, 1971).

Preparation of postmitochondrial supernatant fractions. The rats were killed as a routine between 11:00 and 12:00h. Liver postmitochondrial supernatant fractions were then prepared as described by Richardson *et al.* (1971) except that GSH was omitted from the homogenizing medium and the supernatant fractions were not passed through Sephadex G-25 columns. The fractions were used as soon as possible after preparation.

Incubation procedures. Two incubation mixtures were prepared. The first, referred to in the text, Table 1 and Fig. 1 as the preincubation medium, contained in a volume of 1 ml: 5 mM-MgCl₂, 0.1 M-Hepes buffer, adjusted to pH 7.8 at 20°C with KOH solution, and 0.2 ml of postmitochondrial supernatant (equivalent to 67 mg wet wt. of liver). As indicated in Table 1 and Fig. 1, in various experiments NADP⁺, NAD⁺, glucose 6-phosphate and compound SKF 525A were additional components of the preincubation medium. To introduce 4-dimethylamino-3'-methylazobenzene into this medium, 20 µl of a saturated solution of the carcinogen in ethanol (approx. 240 nmol) was added with a syringe. Corresponding controls received 20 µl of ethanol.

The second mixture, kept chilled until used, contained in a volume of 1 ml: 5 mM-MgCl₂, 140 mM-KCl, 4 mM-ATP, 20 mM-phosphoenolpyruvate, pyruvate kinase (100 µg/ml), 9.12 µM-L-[1-¹⁴C]leucine (0.5 µCi), 0.1 M-Hepes buffer (pH 7.8, as above) and 0.1 ml of postmitochondrial supernatant. In initial experiments 1 mM-GTP was also included but was sub-

sequently omitted when its absence was found not to alter results.

The preincubation medium was shaken vigorously in a water bath under air at 37°C for 15 min and then chilled. Preliminary experiments have shown that in the presence of NADP⁺ (or NAD⁺) *N*-demethylation of aminopyrine or reductive cleavage of the azo bond of 4-dimethylamino-3'-methylazobenzene was detectable, thus proving the activity of microsomal enzymes in the postmitochondrial supernatant under these conditions.

Amino acid incorporation was determined by combining the preincubation medium with the second mixture and incubating at 37°C for a further 10 min. The final incorporation system thus obtained resembled that of Richardson *et al.* (1971) except where additional components were derived from the various preincubation media.

Determination of ¹⁴C radioactivity in protein. The final incubations were terminated by the addition of 2 ml of ice-cold 10% (w/v) trichloroacetic acid containing 0.1% unlabelled L-leucine. Isolated protein was repeatedly washed (Andrews & Tata, 1971) and finally dissolved in 1 ml of 1M-NaOH. Samples were

counted for radioactivity in a model 3390 Packard Tri-Carb liquid-scintillation spectrometer (efficiency approx. 86%) after inclusion in an acidified toluene-Triton X-100-2,5-diphenyloxazole mixture as previously described (Madsen, 1969). Results are reported as uncorrected c.p.m., since it was found by using internal and external standards that quench variation from sample to sample in this system is negligible.

Determination of RNA content of postmitochondrial supernatants. The c.p.m. were related to the RNA content of the volume of supernatant present in the final incorporation system (0.3 ml). RNA was determined by the Schmidt-Thannhauser method as described by Blobel & Potter (1968).

Statistical analysis. A *t* test for matched pairs (Scheffler, 1969) was used to assess the significance of the difference between the means in Expt. 1, Table 1.

Results

The influence of the inclusion in the preincubation medium of 4-dimethylamino-3'-methylazobenzene, and of some substances which enhance or inhibit microsomal drug metabolism, on subsequent cell-free

Table 1. *Effect of preincubated NADP⁺, NAD⁺, glucose 6-phosphate, the carcinogen 4-dimethylamino-3'-methylazobenzene or compound SKF 525A on hepatic cell-free L-[1-¹⁴C]leucine incorporation into protein*

A buffered mixture (preincubation medium; for details see the text) in a final volume of 1 ml was shaken at 37°C for 15 min. Where indicated, the following were added: 1 mM-NADP⁺, 1 mM-NAD⁺, 5 mM-glucose 6-phosphate, 1 mM-compound SKF 525A. The vehicle for addition of 4-dimethylamino-3'-methylazobenzene was 20 µl of ethanol which contained approx. 240 nmol of the carcinogen. When the carcinogen was absent, 20 µl of ethanol alone was added to the samples. After chilling, the samples were combined with a second prepared mixture, whose contents are described in the text, to give a system (total volume 2 ml) supporting the incorporation of L-[1-¹⁴C]leucine into protein. The combined mixtures were incubated at 37°C for 10 min. Liver postmitochondrial supernatant fractions, which were freshly prepared from different rats for the different determinations, supplied components essential for potential metabolism of xenobiotics and for protein synthesis. Protein was isolated, washed and radioactivity of samples determined by liquid-scintillation counting. In Expt. 1, values given are means ± S.E.M. The numbers in parentheses represent the numbers of separate determinations each carried out in duplicate. Mean values of determinations performed in duplicate are shown for Expts. 2, 3 and 4. In each case, the result was typical of two or three separate determinations.

Expt. no.	Additions	Presence of carcinogen ...	10 ⁻² × Radioactivity (c.p.m./mg of RNA)		% change from respective controls	
			Absent	Present	Absent	Present
1	None (10)		146 ± 14	137 ± 12		
	NADP ⁺ + glucose 6-phosphate (10)		263 ± 13*	196 ± 10*	+82	+43
2	None		135	120		
	NADP ⁺ + glucose 6-phosphate		266	191	+96	+59
	NADP ⁺		256	196	+90	+64
	Glucose 6-phosphate		131	128	-3	+7
3	None		204	192		
	NADP ⁺ + glucose 6-phosphate		312	221	+53	+15
	NAD ⁺		325	234	+59	+22
	Glucose 6-phosphate		188	179	-8	-7
4	None		215	207		
	Compound SKF 525A		195	194	-9	-7
	NADP ⁺ + glucose 6-phosphate		348	257	+62	+24
	NADP ⁺ + glucose 6-phosphate + compound SKF 525A		330	236	+53	+14

* Values different from respective controls, *P* < 0.0025.

Fig. 1. Stimulation of cell-free L-[1-¹⁴C]leucine incorporation into protein by various amounts of preincubated NADP⁺ in the absence and presence of the carcinogen 4-dimethylamino-3'-methylazobenzene

The procedures for preincubation and measurement of amino acid incorporation were the same as outlined in Table 1; details of mixtures are described in the text. The amounts of NADP⁺ added to the preincubation media were varied as indicated. Two separate determinations with liver preparations from different rats were performed in duplicate. The pattern of results was similar on each occasion. Mean values from one of the determinations are shown. □, Carcinogen absent; ○, carcinogen present (approx. 240nmol).

incorporation of ¹⁴C-labelled amino acid into protein is shown by the results in Table 1. A noteworthy finding was that NADP⁺ + glucose 6-phosphate usually stimulated incorporation by at least 80% (Expt. 1). This degree of stimulation was decreased to 43% when the carcinogen was also present. The data may be viewed from another angle: the carcinogen decreased incorporation by only 6% in the absence, but by 26% in the presence, of NADP⁺ + glucose 6-phosphate. Expt. 2 revealed that the stimulatory effect and typical response to the carcinogen could be attributed solely to NADP⁺ and not to glucose 6-phosphate. Equimolar NAD⁺ alone was also active (Expt. 3), though not to the degree shown by NADP⁺. In experiments where the effects of NADP⁺ and NAD⁺ were directly compared (results not shown), the stimulatory effect of NAD⁺ was 55% of that of NADP⁺. To test a possible role of mixed-function oxidases in the effect of the carcinogen, in Expt. 4 compound SKF 525A was also included, where shown, in the preincubation media. The presence of this inhibitor of many microsomal reactions failed to alter the basic pattern of stimulated incorporation with NADP⁺ which was decreased by the carcinogen.

The extent of stimulation of incorporation as a function of initial NADP⁺ concentration was investigated. It was found (Fig. 1) that stimulation was detected with the lowest amount added (0.25 μmol) and that maximum stimulation was obtained with 1 μmol. Amounts of NADP⁺ greater than 0.5 μmol were required before the presence of the carcinogen could significantly decrease incorporation.

Discussion

Several lines of investigation have resulted from this attempt to link carcinogen biotransformation *in vitro* with cell-free protein synthesis. Of major interest is the possibility that, if the effects *in vitro* bear any relation to the intracellular situation, the nicotinamide nucleotides may have a regulatory role in protein synthesis. It first remains to be shown in the system *in vitro* whether the stimulatory effects observed on ¹⁴C-labelled amino acid incorporation are specifically caused by the oxidized or reduced forms of the nucleotides or their degradation products.

Associated with these studies should be attempts to identify the site(s) of protein synthesis susceptible to the influence of the nicotinamide nucleotides. This work would be aided by the considerable information now available on the mechanisms by which eukaryotic protein synthesis may be altered (Vazquez, 1974).

The nature of the influence of 4-dimethylamino-3'-methylazobenzene in decreasing the stimulatory effect of nicotinamide nucleotides on cell-free protein synthesis is equally intriguing. Since the presence of compound SKF 525A failed to alter the effect of the carcinogen, it appears that none of the potential metabolites of the carcinogen whose formation is sensitive to the inhibitor could be the active agent. Alternative metabolites such as *N*-oxides or the products of reductive cleavage may be implicated.

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