Effect of some Polycyclic Aromatic Hydrocarbons on Protein Synthesis *in vitro*

By J. HRADEC

Department of Biochemistry, Oncological Institute, Prague 8, Czechoslovakia

(Received 9 January 1967)

1. The effect of the strongly carcinogenic polycyclic aromatic hydrocarbons benzo[*a*]pyrene, 3-methylcholanthrene and dibenz[*a,h*]anthracene and of the non-carcinogenic anthracene, pyrene and phenanthrene on protein synthesis was studied *in vitro* with subcellular systems from rat liver. 2. Both types of hydrocarbons affect amino acid activation and inhibit transfer of labelled amino acids from transfer RNA to ribosomes. 3. Only the carcinogenic compounds stimulate the incorporation of labelled algal-protein hydrolysate and of some individual amino acids into transfer RNA. The most active dose was 10 μ g. under the conditions used. This effect is abolished by preincubation of pH 5 enzymes with the carcinogens before the addition of radioactive amino acids. 4. The carcinogens stimulate the incorporation of some amino acids into ribosomal protein whereas the non-carcinogenic compounds have no such effects. 5. Polynucleotide-dependent stimulation of protein synthesis is greatly enhanced in the presence of the carcinogenic hydrocarbons when either free amino acids or transfer RNA charged with labelled amino acids are used. The non-carcinogenic compounds induce a partial inhibition of this process. 6. It is concluded, in agreement with other authors, that carcinogens may increase the number of active incorporation sites on both transfer and ribosomal RNA. Possible mechanisms of such an effect are discussed.

There are relatively few papers on the effects of carcinogenic compounds on protein synthesis, although chemical carcinogenesis is probably followed or preceded by changes in these enzymic reactions.

Several authors have reported changes in the protein-synthesizing systems of animals pre-treated with carcinogenic hydrocarbons (Gelboin & Sokoloff, 1961), amines (Hultin & Arrhenius, 1965), or azo-dyes (Hawtrey & Schirren, 1962). However, only Chang & Bond (1964) used a system *in vitro* suitable for a study of the direct effects of carcinogenic hydrocarbons on reactions involved in protein synthesis. After additions of relatively high quantities of 7,12-dimethylbenz[*a*]anthracene and 3-methylcholanthrene into the incubation mixtures, they obtained a decreased incorporation of labelled glycine and glutamic acid into the s-RNA* of *Escherichia coli*. In a previous paper (Hradec, 1959) an enhanced incorporation of labelled amino acids into the proteins of rat-liver homogenate in the presence of carcinogenic hydrocarbons was described.

The experiments reported in the present paper

* Abbreviations: s-RNA, 'soluble' or transfer RNA; polyU, polyuridylic acid; polyA, polyadenylic acid.

were performed to elucidate at which stage carcinogenic hydrocarbons affect protein synthesis, and to what extent this effect is specific for carcinogenic substances only. Cell-free systems of rat liver were used and the effects of chemically closely related non-carcinogenic hydrocarbons were studied as controls.

MATERIALS AND METHODS

Animals. Wistar rats of both sexes, weighing 150–200 g., bred in this Laboratory and kept on a mixed diet, were used.

Chemicals. All carcinogenic and non-carcinogenic hydrocarbons were products of Koch–Light Laboratories Ltd., Colnbrook, Bucks. Phosphoenolpyruvic acid (silver–barium salt), pyruvate kinase and GTP (sodium salt) were from the same source. Natural L-amino acids were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and polyA and polyU from Calbiochem, Los Angeles, Calif., U.S.A. ATP (sodium salt) was a product of Reanal, Budapest, Hungary. Since this preparation was found to contain some impurities inhibiting protein synthesis it was purified by ion-exchange chromatography by essentially the method of Cohn & Carter (1950).

Radioactive compounds. U-¹⁴C-labelled algal-protein hydrolysate (769 mc/g.), L-[U-¹⁴C]leucine (72 mc/m-mole), L-[U-¹⁴C]phenylalanine (66 mc/m-mole), L-[U-¹⁴C]valine (51 mc/m-mole), L-[U-¹⁴C]lysine (88 mc/m-mole), L-[U-¹⁴C]-

aspartic acid (62mc/m-mole) and sodium [^{32}P]pyrophosphate (7.1–9.6mc/m-mole) were products of the Institute for Research, Production and Utilization of Radioisotopes, Prague, Czechoslovakia. s-RNA labelled with individual amino acids was prepared as described by Moldave (1963).

Preparation of subcellular fractions. Rats were killed by breaking their necks, and their livers were quickly removed, plunged into ice-cold medium A (Littlefield & Keller, 1957) and chopped with scissors. Pooled liver tissue (usually 240g.) was homogenized in several portions in a Potter-Elvehjem homogenizer fitted with a plastic pestle, 19ml. of ice-cold medium A being added for each 8g. of liver tissue (Kirsch, 1962). The homogenate was centrifuged at 16000g for 10 min. in an 8×50 ml. or a 6×100 ml. rotor of a VAC 40 or a VAC 60 preparative ultracentrifuge (H. Janetzki KG., Engelsdorf/Leipzig, Germany). The supernatants were carefully transferred to other tubes and spun at 105000g for 1 hr. in the 8×50 ml. rotor. The microsomal sediments were treated with deoxycholate according to the procedure of Kirsch (1962). After centrifugation at 105000g for 45 min., the ribosomal pellets were homogenized in the same volume of medium A and spun at 105000g for another 45 min. The supernatants were discarded and the ribosomal pellets stored in the unsuspended state at -50° for 1–2 weeks. Immediately before use the ribosomes were suspended in medium A by gentle homogenization. The postmicrosomal supernatant (S-105) was used without further fractionation in some experiments. In this case it was stored in small portions at -50° as described below. More often, however, it was used for further isolation of pH5 enzymes. If so, it was acidified to pH 5.2 by the addition of N-acetic acid. After 10 min. the resulting precipitate was separated by low-speed centrifugation, the pellets were resuspended by homogenization in half of the original volume of medium A and the pH was brought again to 5.2. The precipitate was collected as before and homogenized in medium A to give a concentration of 50–100mg./ml. The pH5 enzyme suspension was divided into smaller portions, each sufficient for one set of experiments (to avoid repeated thawing and freezing of this preparation), and stored at -50° . For experiments on pyrophosphate exchange, pH5 enzymes were dialysed overnight against distilled water in a cold-room before storage at -50° . The pH5 supernatant was brought to pH 7.60 by the addition of 0.5M-tris, divided into smaller portions in the same way as the pH5 enzymes and stored again at -50° . All procedures described here were carried out at 0– 5° .

Incubation procedures. All reaction mixtures were prepared in conical test tubes immersed in ice-cold water. Samples were then equilibrated for 2 min. at 37.5° , and the reaction was started by the addition of the labelled compound. All samples were incubated in duplicate. Each series contained blank samples, which were samples without sources of energy (ATP, or ATP and an ATP-generating system) or, for the transfer reaction, zero-time samples. A Dubnoff incubator was used for all incubations. Reactions were terminated by immersing the tubes in ice-cold water and immediately adding protein-precipitating agents where indicated.

All the hydrocarbons were added into the incubation mixtures as suspensions in polyethylene glycol (mol.wt. 600). For the preparation of such a suspension a weighed

quantity of the compound was dissolved in a small volume of ether and the necessary amount of polyethylene glycol added. Ether was evaporated by agitating the flask under hot water and last traces of ether were eliminated with a stream of nitrogen. In this way homogeneous and stable suspensions could be prepared at concentrations up to 1mg./ml.

Incubation mixtures used for the assay of the ATP-pyrophosphate exchange reaction were the same as those described by Stulberg & Novelli (1962). When the incorporation of labelled amino acids into s-RNA was studied, incubation mixtures contained 2ml. of medium A, 0.1ml. of pH5 enzyme suspension (5–10mg. of protein), 0.1ml. of hydrocarbon suspension (or 0.1ml. of polyethylene glycol in control experiments), 10 μ moles of ATP and 7–8 μ moles of labelled amino acid or 0.6 μ g. of protein hydrolysate in a total volume of 2.4 ml. They were incubated for 10 min. at 37.5° .

The systems used for the incorporation of labelled amino acids into ribosomal protein were prepared essentially as described by Weisberger, Armentrout & Wolfe (1963) and consisted of 100 μ moles of tris buffer, pH 7.60, 5 μ moles of Mg^{2+} , 50 μ moles of KCl, 1 μ mole of ATP, 0.25 μ mole of GTP, 10 μ moles of phosphoenolpyruvate, 40 μ g. of pyruvate kinase, a mixture of the 20 natural amino acids (0.04 μ mole of each) without that used in radioactive form (the amino acid mixture was omitted in experiments with labelled protein hydrolysate), 0.1 ml. of hydrocarbon suspension (or 0.1 ml. of polyethylene glycol in control experiments), 0.1 ml. of S-105 fraction (8–10mg. of protein), 0.1 ml. of ribosome suspension (3–5mg. of protein) and 7–8 μ moles of labelled amino acid or 0.6 μ g. of labelled protein hydrolysate in a total volume of 1.0 ml. PolyU or polyA (100 μ g.) was added where indicated. Mixtures were incubated for 60 min. at 37.5° .

For the study of the transfer of labelled amino acids from s-RNA to ribosomes, systems similar to those described by Moldave (1964) were used. Incubation mixtures contained 0.8 ml. of medium A, 10 μ moles of ATP, 0.5 μ mole of GTP, 7 μ moles of Mg^{2+} , 0.2 ml. of pH5 supernatant (10mg. of protein), 0.1 ml. of ribosome suspension (3–5mg. of protein), 0.1 ml. of hydrocarbon suspension (or 1 ml. of polyethylene glycol in control samples) and 0.1–0.2 ml. of labelled amino acid-charged s-RNA (850–1600 counts/min.) in a total volume of 1.6 ml. PolyU or polyA (100 μ g.) was added where indicated. Incubation was carried on for 20 min. at 37.5° .

Preparation of samples for radioactivity assay. In the study of the ATP-pyrophosphate exchange reaction, proteins were precipitated and ATP isolated and hydrolysed as described by Stulberg & Novelli (1962). From the final acid hydrolysate, a portion was taken for the determination of phosphorus and another portion diluted to 10 ml. for counting.

The pH5 enzyme proteins after incubation with labelled amino acids were precipitated and washed as described by Hoagland, Stephenson, Scott, Hecht & Zamecnik (1958). They were then extracted with 0.5 ml. of 0.5N-perchloric acid at 80° for 30 min. After centrifugation a sample of the supernatant was withdrawn for the determination of RNA content and another portion was placed on aluminium planchets and dried at 50° for counting.

Proteins from incubation mixtures containing ribosomes and labelled amino acids were precipitated and washed as

described by Weisberger *et al.* (1963). The final protein precipitate was suspended by homogenization in ethanol and plated on Whatman no. 3 filter-paper disks by suction. Disks with protein precipitates were then fixed on to aluminium planchets and counted.

Incubation mixtures in experiments where the transfer reaction was followed were diluted to 10ml. with ice-cold medium A after incubation. They were spun at 105000g for 45 min. The supernatant was discarded and the ribosomes were homogenized in medium A and centrifuged again under the same conditions. The ribosomal pellet was suspended in a small volume of medium A and the proteins were precipitated and washed by the procedure of Moldave (1960). Samples of ribosomal proteins for radioactivity assay were prepared in the same way as described in the preceding paragraph.

Radioactivity assay. Solutions containing ^{32}P were poured into polyacrylate scintillation vials and counted in an NBZ 412 liquid-scintillation spectrometer with 25% counting efficiency. RNA and proteins were counted as infinitely thin samples with an NZR 211 windowless gas-flow counter connected to an NZQ 615 automatic scaler. Counting efficiency was 30% for RNA and about 22% for proteins. Samples were counted for sufficient periods to decrease the probable error to less than $\pm 2\%$. The results were corrected for background and self-absorption, when necessary, by using an experimentally determined curve obtained with proteins of known specific activity. All counting equipment was supplied by Tesla, Přemyslení, nr. Prague, Czechoslovakia.

Chemical determinations. Proteins were determined by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951), inorganic phosphorus by the method of Fiske & Subbarow (1925) and RNA on the basis of its E_{260} , with a conversion factor 24.0 (Webster & Davie, 1961). All extinctions were measured with a VSU-1 Universal Spectrophotometer (Carl Zeiss, Jena, Germany).

RESULTS

Amino acid activation. Widely differing results were obtained in experiments to study the effect of

hydrocarbons on the first step of protein synthesis when different amino acids were used as substrates.

When a complete mixture of 20 natural amino acids (mixture A) was added to pH 5 enzymes, an ATP-pyrophosphate exchange (Stulberg & Novelli, 1962) of 2.33–2.51% (lowest and highest value of four experiments) was obtained in control samples. All the hydrocarbons tested induced a partial inhibition of this process (0.90–1.80% exchange).

Rather different results were found when tryptophan was used as substrate. In this case the control value was 0.35–0.42% (three experiments). When carcinogenic hydrocarbons (benzopyrene, methylcholanthrene and dibenzanthracene) were added into the incubation mixture the exchange was 0.35–0.95%, whereas in the presence of non-carcinogenic compounds 0.25–0.40% exchange was found. The differences between these values and those obtained in control samples were not significant ($P < 0.1$ and $P < 0.4$, respectively).

With amino acid mixture B, consisting of phenylalanine, lysine, leucine and valine, control values of 0.67–0.87% exchange were obtained. Although benzopyrene, methylcholanthrene and, to a smaller extent, also pyrene stimulated exchange with these amino acids (1.01–1.90%), all other hydrocarbons had a slight inhibitory effect, which was, however, not significant ($P < 0.2$).

Distinct differences between the effects of carcinogenic and non-carcinogenic compounds were again not found when amino acid mixture C (histidine, isoleucine, serine and alanine) was added. The control value was 1.32–1.53% (four experiments). All carcinogenic hydrocarbons as well as anthracene showed a significant ($P < 0.01$) stimulatory effect (1.32–1.92% exchange), but pyrene and phenanthrene caused an inhibition (0.85–1.25% exchange).

Table 1. *Effect of polycyclic aromatic hydrocarbons on amino acid activation*

The amount of ATP-pyrophosphate exchange is used as a measure of amino acid activation (see the text). All values are percentages of the control values from the samples (incubated with polyethylene glycol only). They were calculated for each complete set of experiments consisting of blank and control incubation mixtures together with mixtures containing the compounds tested. Average values from three to five such series of experiments are given. Incubation conditions were as described in the Materials and Methods section. The amino acid mixture A contained all 20 natural amino acids; the compositions of mixtures B and C are given in the Results section. Hydrocarbons (10 μg .) were added to incubation mixtures where indicated.

Amino acid added ... Hydrocarbon	Amino acid activation (% of control)			
	Tryptophan	Mixture A	Mixture B	Mixture C
Benzo[a]pyrene	208	77	283	123
3-Methylcholanthrene	271	50	178	165
Dibenz[a,h]anthracene	100	70	45	102
Anthracene	96	90	83	125
Pyrene	60	56	116	82
Phenanthrene	76	38	57	56

Fig. 1. Effect of various doses of benzo[a]pyrene (○) and anthracene (●) on the incorporation of ^{14}C -labelled algal-protein hydrolysate into s-RNA. The control value is indicated by the broken line (-----).

Fig. 2. Incorporation of ^{14}C -labelled L-lysine into s-RNA in control samples (●) and into mixtures containing $10\text{m}\mu\text{g.}$ of benzo[a]pyrene (○).

A summary of the results obtained in experiments on amino acid activation is given in Table 1.

Incorporation of amino acids into s-RNA. Addition of hydrocarbons in various concentrations to mixtures containing pH5 enzymes, ATP and labelled protein hydrolysate showed a rather curious relation between the dose and the effect when carcinogenic substances were used. All the carcinogenic compounds induced maximal stimulation of this reaction when a dose of $10\text{m}\mu\text{g.}$ was used, higher as well as lower amounts being less effective. However, when the non-carcinogenic hydrocarbons were used, the maximal stimulatory effect was observed with the highest dose, followed by a progressive decrease of effect with lower doses. With anthracene, however, only the two highest doses used had a significantly different value when compared with control mixtures ($P < 0.01$ and $P < 0.05$ respectively). These results are shown in Fig. 1.

A study of the time-course of incorporation of labelled protein hydrolysate, as well as some individual amino acids, into s-RNA showed a basic difference between control mixtures and those into which carcinogenic hydrocarbons were added. Higher radioactivity was found in the s-RNA of mixtures containing carcinogens at all intervals studied, and the maximal value was reached after 8 min. of incubation. This means that the s-RNA is

more rapidly charged with at least some amino acids in the presence of carcinogens. The results with labelled lysine are given in Fig. 2. Similar results were also obtained when labelled protein hydrolysate or aspartic acid was used.

A systematic study of the effect of hydrocarbons on the activity of various amino acyl-s-RNA synthetases again revealed differences between individual enzymes. A concentration of $10\text{m}\mu\text{g.}$ of hydrocarbons was used in all these experiments.

When labelled protein hydrolysate was used as the source of amino acids, the net energy-dependent incorporation into s-RNA in control mixtures was 185–310 counts/min./mg. of s-RNA (lowest and highest value of five identical experiments with various batches of pH5 enzymes). Carcinogenic hydrocarbons greatly stimulated the incorporation of labelled amino acids in this system whereas non-carcinogenic compounds had an inhibitory effect or no effect at all.

However, no such clear differences were found in systems containing some individual amino acids instead of protein hydrolysate. All the hydrocarbons tested were essentially without effect on the incorporation of labelled phenylalanine into s-RNA (control value 580–710 counts/min./mg. of s-RNA, four experiments). On the other hand, the charging of s-RNA with lysine was slightly stimulated in the

Table 2. *Effect of polycyclic aromatic hydrocarbons on the incorporation of labelled amino acids into s-RNA*

All values are average percentages of the control value as explained in Table 1. Incubation conditions were as described in the Materials and Methods section. Hydrocarbons (10 μ g.) were added to mixtures where indicated.

Labelled amino acid added	Incorporation into s-RNA (% of control)					
	Hydrolysate	Leucine	Valine	Phenylalanine	Lysine	Aspartic acid
Hydrocarbon						
Benzopyrene	385	108	110	105	130	158
Methylcholanthrene	407	72	98	95	135	77
Dibenzanthracene	284	101	105	92	132	53
Anthracene	91	98	86	84	121	128
Pyrene	70	80	100	84	118	33
Phenanthrene	101	117	97	90	154	103

presence of all the hydrocarbons used (control value 184–275 counts/min./mg. of s-RNA, four experiments). Incorporation of leucine (483–620 counts/min./mg. of s-RNA, three experiments), valine (257–430 counts/min./mg. of s-RNA, five experiments) and aspartic acid (230–350 counts/min./mg. of s-RNA, three experiments) was in some cases stimulated and in others inhibited, but no differences were found in this respect between carcinogenic and non-carcinogenic compounds (Table 2).

Unexpected differences between the effects of hydrocarbons on the incorporation of individual amino acids and of the protein hydrolysate suggested that the presence of a complete amino acid mixture might be necessary for maximal effect. Mixtures of the 20 natural amino acids (omitting that used in radioactive form) were therefore added to incubation mixtures in further experiments. This treatment did not significantly affect the incorporation in control samples. However, the activity of some individual amino acyl-s-RNA synthetases was affected by this addition. Although incorporation of labelled phenylalanine into s-RNA was not significantly influenced by the presence of hydrocarbons in the previous experiments, after addition of the amino acid mixture results corresponding to those obtained with protein hydrolysate were found; the carcinogenic hydrocarbons stimulated this process, whereas the non-carcinogenic compounds were without effect. With valine, the carcinogens were without effect but the non-carcinogenic compounds were inhibitory. However, addition of amino acids had no effect when lysine or aspartic acid was used.

Mixtures containing pH5 enzymes and ATP were preincubated at 37.5° with hydrocarbons before labelled protein hydrolysate was added. This treatment had a significant effect on the stimulating action of the carcinogenic substances. Less stimulation was found after longer periods of preincubation and after 10–20 min. the stimulating

Table 3. *Effect of polycyclic aromatic hydrocarbons on the incorporation of labelled phenylalanine and valine into s-RNA in the presence of a complete amino acid mixture*

All values are average percentages of the control value as explained in Table 1. Incubation conditions were as described in the Materials and Methods section. Each incubation mixture contained a complete mixture of 20 natural amino acids (0.04 μ mole of each) omitting that added in radioactive form. Hydrocarbons (10 μ g.) were added to mixtures where indicated.

Labelled amino acid used	Incorporation into s-RNA (% of control)	
	Phenylalanine	Valine
Hydrocarbon		
Benzopyrene	172	108
Methylcholanthrene	149	101
Dibenzanthracene	158	98
Anthracene	100	75
Pyrene	105	94
Phenanthrene	102	90

ability of the carcinogens was completely abolished. Moreover, similar results were also obtained if 100-fold higher doses of these substances were used, suggesting that this effect is unlikely to be due to a rapid degradation of carcinogenic compounds during preincubation. Preincubation of pH5 enzymes with non-carcinogenic compounds had no such effect. Some of these results are shown in Fig. 3.

Aminoacyl transfer from s-RNA to ribosomes. All hydrocarbons tested had an inhibitory effect on this second step of protein synthesis. This effect seems to be rather unspecific, since the highest doses of both carcinogenic and non-carcinogenic compounds showed the greatest effect; the effect was less when lower doses were used. However, concentrations as low as 0.1 μ g. were able to inhibit

Fig. 3. Incorporation of ¹⁴C-labelled protein hydrolysate into s-RNA in preincubated control mixtures (○), those containing 1 μg. (■) or 10 μg. of dibenz[a,h]anthracene (●), or 10 μg. of pyrene (□). Samples were preincubated in the presence of the hydrocarbons before the addition of labelled hydrolysate for the periods indicated.

Fig. 4. Effect of various doses of 3-methylcholanthrene (○) and anthracene (●) on the transfer of ¹⁴C-labelled L-lysine from s-RNA to ribosomes. The control value is indicated by the broken line (-----).

the transfer reaction. These results are shown in Fig. 4.

Similar inhibitions were induced by both classes

Table 4. *Effect of polycyclic aromatic hydrocarbons on aminoacyl transfer from s-RNA to ribosomes*

All values are percentages of the control value as explained in Table 1. The total radioactivity added to incubation mixtures when leucyl-, lysyl- and phenylalanyl-s-RNA were used was 1600 counts/min., 850 counts/min. and 865 counts/min. respectively. Incubation procedures were as described in the Materials and Methods section. Hydrocarbons (10 μg.) were added where indicated.

s-RNA charged with ... Hydrocarbon	Amino acyl transfer (% of control)		
	Leucine	Lysine	Phenylalanine
Benzopyrene	87	64	77
Methylcholanthrene	77	70	81
Dibenzanthracene	60	77	81
Anthracene	53	44	81
Pyrene	55	44	72
Phenanthrene	58	47	80

Table 5. *Effect of polycyclic aromatic hydrocarbons on the polynucleotide-directed transfer of amino acids from s-RNA to ribosomal protein*

All values are average percentages (see Table 1) of differences between the samples with and without polynucleotides added to control mixtures. Incubation conditions were as described in the Materials and Methods section. Phenylalanine was added to mixtures containing polyU and lysine to those with polyA. Hydrocarbons (10 μg.) were added where indicated.

s-RNA charged with ... Hydrocarbon	Difference in incorporation between samples with and without polynucleotide (% of difference in control expt.)	
	Phenylalanine	Lysine
Benzopyrene	159	176
Methylcholanthrene	115	450
Dibenzanthracene	200	353
Anthracene	39	41
Pyrene	17	73
Phenanthrene	75	30

of compounds when s-RNA charged with labelled phenylalanine was used. The net radioactivity of ribosomal proteins, after subtraction of the zero-time value in control samples of three experiments with different batches of ribosomes and cellular supernatant, was 18.1–21.3 counts/min./mg. of protein. For leucyl- and lysyl-s-RNA (control values 47.5–60.6 and 15.5–20.7 counts/min./mg. of protein respectively), however, some differences seem to exist between the carcinogenic and non-carcinogenic substances, although both these classes of compounds cause an inhibition of transfer. This

Table 6. *Effect of polycyclic aromatic hydrocarbons on the incorporation of labelled amino acids into ribosomal protein*

All values are average percentages of the control values as described in Table 1. Incubation procedures are given in the Materials and Methods section. Hydrocarbons (10 μ g.) were added to incubation mixtures where indicated. —, Not determined.

Labelled amino acid Hydrocarbon	...	Incorporation into protein (% of control)			
		Hydrolysate	Lysine	Leucine	Phenylalanine
Benzopyrene		310	189	103	193
Methylcholanthrene		127	155	98	144
Dibenzanthracene		149	122	98	142
Anthracene		116	41	110	—
Pyrene		118	35	103	85
Phenanthrene		121	30	99	98

inhibitory activity is much more pronounced with non-carcinogenic than with carcinogenic hydrocarbons. These results are shown in Table 4, where the total radioactivities used in experiments with different types of s-RNA are also shown.

The addition of synthetic polynucleotides to mixtures incubated with phenylalanyl- or lysyl-s-RNA greatly enhanced the incorporation of amino acids into ribosomal protein in both control and experimental mixtures. In control samples the specific activities of ribosomal protein were about twice as high in the presence of polyU or polyA as in the absence of these polynucleotides. The difference between the values found for samples incubated with and without polyU in the presence of strong carcinogens was, however, greater than in control mixtures. On the other hand, all three non-carcinogenic compounds tested showed a partial inhibition of polyU-dependent stimulation of phenylalanine transfer when compared with controls. A similar enhancement of polyA-directed polylysine formation was demonstrated in mixtures containing the carcinogens. The difference between samples with and without polyA to which the non-carcinogenic substances were added was again lower than in the control mixtures (Table 5).

Incorporation of labelled amino acids into ribosomes. The presence of any of the compounds tested stimulated the incorporation of labelled protein hydrolysate in the complete subcellular protein-synthesizing system. The carcinogens were more active in this respect than the non-carcinogenic hydrocarbons. Net specific activity (after subtraction of the values from blank samples incubated without ATP and an ATP-generating system) of ribosomal protein in control mixtures containing algal-protein hydrolysate was 240–380 counts/min./mg. of protein (lowest and highest values from four experiments with various batches of subcellular fractions). Incorporation of radioactive lysine (control value 41–54 counts/min./mg. of protein,

three experiments) into ribosomal protein was enhanced by the addition of the three powerful carcinogens, whereas the non-carcinogenic compounds inhibited this process. Similar results were obtained with phenylalanine (control value 105–215 counts/min./mg. of protein, four experiments). On the other hand, the hydrocarbons did not affect the incorporation of leucine (185–315 counts/min./mg. of protein, three experiments) into ribosomal protein to any significant extent (Table 6).

The complete subcellular system used in this study gave a good response to synthetic polynucleotides. In control mixtures addition of polyU increased the incorporation of labelled phenylalanine into ribosomal protein 5.2–5.5-fold (three experiments), and the incorporation of lysine was stimulated 2.4–3.6-fold in the presence of polyA (four experiments). Mixtures containing both hydrocarbons and polynucleotides gave similar results to those described in the transfer reaction. The difference in incorporation between the samples with and without polyU or polyA was again greater in samples containing the carcinogens than in the control mixtures. Similarly, the non-carcinogenic compounds inhibited to some extent the polynucleotide-directed synthesis of polyphenylalanine and polylysine. These results are given in Table 7.

DISCUSSION

A rather strange dose-effect relationship emerged when carcinogens were added to most of the incubation mixtures used in this study. This relationship is very similar to that obtained with the endogenous carcinogen Carcinolipin (Hradec, 1961). The latter substance, however, can stimulate all steps of protein synthesis.

The observations that the effects of carcinogens may vary with different amino acids agree well with results reported by Chang & Bond (1964), who describe a decrease of aminoacyl-s-RNA synthetase

Table 7. *Effect of polycyclic aromatic hydrocarbons on polynucleotide-dependent incorporation of labelled phenylalanine and lysine into ribosomal protein*

All values are average percentages of differences between the samples with and without polynucleotides added to control mixtures (see Table 5). Incubation conditions were as described in the Materials and Methods section. Phenylalanine was added to mixtures containing polyU and lysine to those with polyA. Hydrocarbons (10 μ g.) were added to mixtures where indicated.

Amino acid used ... Hydrocarbon	Difference in incorporation between samples with and without polynucleotide (% of the difference in control expt.)	
	Phenylalanine	Lysine
Benzopyrene	122	237
Methylcholanthrene	169	155
Dibenzanthracene	357	137
Anthracene	100	95
Pyrene	59	33
Phenanthrene	95	43

activity in *E. coli* grown in the presence of carcinogens. They conclude that these compounds affect this enzymic system rather than s-RNA. However, this explanation seems inconsistent with our present experiments. Preincubation of pH 5 enzymes with the carcinogenic hydrocarbons resulted in a complete loss of their ability to affect amino acid incorporation. Moreover, amino acid activation and charging of s-RNA with amino acids is generally believed to represent two aspects of the same enzymic reaction (Hoagland *et al.* 1958), but differences between these two actions appeared in our experiments. It therefore seems more likely that carcinogenic hydrocarbons may affect the capacity of s-RNA to accept individual amino acids in a similar way to that in which the carcinogens probably affect binding sites for amino acids on ribosomal RNA (see below). Differences in the experimental systems used, i.e. bacterial or mammalian, may also explain the observed differences from the results of Chang & Bond (1964). Moreover, Tween, which was used by these authors, is not a completely inert solvent in protein-synthesizing systems, as was revealed by preliminary experiments to find the most suitable solvent for hydrocarbons.

The stimulating effects of the carcinogens in systems containing ribosomes seem to indicate that carcinogens may 'activate' ribosomes by increasing the number of their incorporation sites available for amino acids. This suggestion is in good agreement with the results of other authors using different experimental systems. Gelboin (1964) concluded that the increased incorporation of

amino acids into liver microsomes of rats pretreated with 3-methylcholanthrene is in part due to an increase in the number of active microsomal incorporation sites. Arrhenius & Hultin (1962) supposed that increased protein synthesis in liver microsomes of rats given 2-aminofluorene *in vivo* may be caused by a higher activity of ribonucleoprotein particles. Similarly, Jondorf, Spector & Chaiken (1965) assumed that stimulation of protein synthesis in liver microsomes of rats pretreated with 3-methylcholanthrene may involve an effect on the sensitivity of microsomal systems to s-RNA.

It is not clear how this 'activation' of ribosomes may occur, however. Increased RNA synthesis is probably not the reason for this effect. Gelboin & Blackburn (1963) supposed that the stimulatory effect of methylcholanthrene on protein synthesis is due to an increased production of messenger RNA. However, Bresnick, Knight & Madix (1966) failed to confirm such an enhanced synthesis of messenger RNA, and a decrease of RNA synthesis resulted from the administration of 7,12-dimethylbenz[*a*]anthracene to rats (Libby & Dao, 1966). Moreover, Bresnick, Brand & Knight (1966) did not find changes in the incorporation of labelled orotic acid into RNA in rats pretreated with methylcholanthrene.

Direct binding of carcinogenic hydrocarbons to RNA may be another mechanism by which the activity of RNA might be altered. It has been shown that, after local administration, carcinogenic hydrocarbons become firmly bound not only to proteins (Heidelberger, 1964) but also to DNA and RNA (Oehlert & Grimm, 1966). It is not known, however, whether this binding plays any role in protein synthesis.

There are differences in the effects of carcinogenic hydrocarbons not only on the incorporation of different amino acids but also on the incorporation of a particular amino acid at various stages of protein synthesis. It is apparent that the interference of carcinogenic substances in protein synthesis is very complex and that effects on RNA, although most probable, should not be taken to be the only mechanism by which carcinogens affect protein synthesis.

I am indebted to Dr Z. Dušek for his collaboration in the isolation of subcellular fractions. The highly skilled technical assistance of Mrs Z. Tuháková and Miss N. Krejčová is also gratefully acknowledged.

REFERENCES

- Arrhenius, E. & Hultin, T. (1962). *Cancer Res.* **22**, 823.
- Bresnick, E., Brand, R. & Knight, J. A. (1966). *Biochim. biophys. Acta*, **114**, 227.
- Bresnick, E., Knight, J. A. & Madix, J. C. (1966). *Biochim. biophys. Acta*, **119**, 624.

- Chang, M. & Bond, T. J. (1964). *Nature, Lond.*, **201**, 623.
- Cohn, W. E. & Carter, C. E. (1950). *J. Amer. chem. Soc.* **72**, 2606.
- Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
- Gelboin, H. V. (1964). *Biochim. biophys. Acta*, **91**, 130.
- Gelboin, H. V. & Blackburn, N. R. (1963). *Biochim. biophys. Acta*, **72**, 657.
- Gelboin, H. V. & Sokoloff, L. (1961). *Science*, **134**, 611.
- Hawtrey, A. O. & Schirren, V. (1962). *Biochim. biophys. Acta*, **55**, 785.
- Heidelberger, C. (1964). *J. cell. comp. Physiol.* **64**, Suppl. 1, 129.
- Hoagland, M. B., Stephenson, M. L., Scott, J. F., Hecht, L. I. & Zamecnik, P. C. (1958). *J. biol. Chem.* **231**, 241.
- Hradec, J. (1959). *Brit. J. Cancer*, **13**, 336.
- Hradec, J. (1961). *Biochim. biophys. Acta*, **47**, 149.
- Hultin, T. & Arrhenius, E. (1965). *Cancer Res.* **25**, 124.
- Jondorf, W. R., Spector, A. & Chaiken, S. J. (1965). *Biochem. biophys. Res. Commun.* **20**, 787.
- Kirsch, J. F. (1962). *Biochim. biophys. Acta*, **55**, 541.
- Libby, P. R. & Dao, T. L. (1966). *Science*, **153**, 303.
- Littlefield, J. W. & Keller, E. B. (1957). *J. biol. Chem.* **224**, 13.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Moldave, K. (1960). *J. biol. Chem.* **235**, 2365.
- Moldave, K. (1963). In *Methods in Enzymology*, vol. 6, p. 757. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Moldave, K. (1964). *Advanc. Chem. Ser.* **44**, 41.
- Oehlert, W. & Grimm, D. (1966). *Z. Krebsforsch.* **68**, 14.
- Stulberg, M. P. & Novelli, G. D. (1962). In *Methods in Enzymology*, vol. 5, p. 703. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Webster, L. T. & Davie, E. W. (1961). *J. biol. Chem.* **236**, 479.
- Weisberger, A. S., Armentrout, S. & Wolfe, S. (1963). *Proc. nat. Acad. Sci., Wash.*, **50**, 86.