It is a pleasure to thank Dr C. Long for giving details of the method of sulphatide analysis before publication, and Dr G. H. Sloane-Stanley for helpful discussions on the washing procedure used for removal of radioactive sulphate and for a gift of sulphatide A.

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Stimulation of Ascorbic Acid Synthesis and Excretion by Carcinogenic and other Foreign Compounds

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(*Received* 21 *March* 1961)

Increases in the hepatic ascorbic acid concentrations of mice occur after a single injection of a variety of carcinogenic hydrocarbons (Kennaway, Kennaway & Warren, 1944; Daff, Hoch-Ligeti, Kennaway & Tipler, 1948) although experiments with smaller doses of some carcinogenic compounds had not shown such increases (Boyland & Mawson, 1938).

Longenecker, Fricke & King (1940) demonstrated that narcotics such as chloretone raise the excretion of ascorbic acid in rats and considered that this was associated with an increase in detoxication processes. Chloretone or barbital treatment stimulates the biosynthesis of glucuronic acid and ascorbic acid (Burns & Evans, 1956; Burns, Evans & Trousof, 1957; Evans, Conney, Trousof & Burns, 1960). This increase is abolished by hypophysectomy but not by adrenalectomy (Conney, Gastel & Burns, 1959), nor is it an effect of the drug on the mechanism for the renal tubular reabsorption of ascorbic acid since it can also be produced in nephrectomized rats (Burns *et al.* 1960). Increase in ascorbic acid excretion also occurs after the injection of aminopyrine, antipyrine, diphenhydramine, chlorcyclizine, orphenadrine, meprobamate and phenylbutazone (Burns *et al.* 1960). These authors suggest that the raised biosynthesis of ascorbic acid that takes place after an injection of a wide variety of chemical compounds represents an adaptive response to foreign compounds although borneol and aniline, which are conjugated with glucuronic acid, do not affect ascorbic acid excretion. An increase in ascorbic acid excretion after treatment with carcinogenic hydrocarbons has

been noted by Allen & Boyland (1957) and by Conney *et al.* (1959).

The precise mechanism by which foreign compounds affect glucuronic and ascorbic acid biosynthesis is not known, but the response to some foreign substances is not restricted entirely to an increase in ascorbic acid production; increases in the activity of hepatic microsomal enzymes that metabolize foreign substances also occur after the administration of some hypnotics and carcinogenic hydrocarbons (Conney, Miller & Miller, 1956; Conney *et al.* 1959; Inscoe & Axelrod, 1960).

The present work describes the effect on ascorbic acid metabolism of a variety of foreign compounds.

MATERIALS AND METHODS

D-Galacturonic acid was purchased from L. Light and Co. Ltd., Colnbrook, Bucks., and the sodium salt of 2:6-dichlorophenol-indophenol from Hopkin and Williams Ltd. The extracts of quebracho and mimosa were gifts from Dr K. S. Kirby. Other chemicals used were of analytical grade; reagents were prepared in water distilled in glass. Dextran C and Imferon were from Benger's Ltd., Holmes Chapel, Cheshire.

Slonaker rats were obtained from Dr A. L. Walpole, Imperial Chemical Industries (Pharmaceutical Division) Ltd., Alderley Park, Cheshire.

Preparation of L-*galactono-γ-lactone.* D-Galacturonic acid (4 g.) was dissolved in methanol (100 ml.), Raney Ni catalyst (1·0 g.) was added, and the mixture heated at 100° for 24 hr. under a pressure of 150 atm. of H_2 in a Baskerville rocking autoclave. The cooled mixture was treated with activated charcoal and filtered. The filtrate was evaporated to dryness under reduced pressure, and the syrup transferred, with the aid of a little hot methanol, to a dish which was kept *in vacuo* over conc. H_2SO_4 for 48 hr. The white waxy solid (3·2 g.) was crystallized from absolute ethanol–ether (5:1, v/v) to give 2·1 g. of the lactone (55% of theory), m.p. 128°.

Excretion of ascorbic acid. Pairs of 200 g. male rats were housed in metabolism cages designed for the separate collection of urine and faeces and were maintained on a constant 20% protein diet throughout. All-glass urine separators were used and urine collections were made in flasks containing 10 ml. of aq. metaphosphoric acid (12%, w/v).

Estimation of ascorbic acid. This was estimated by direct titration with a solution of the sodium salt of 2:6-dichlorophenol-indophenol (Harris & Olliver, 1942). In some experiments 'total' ascorbic acid, which includes dehydroascorbic acid and 2:3-dioxogulonic acid, was determined by the method of Roe & Kuether (1943) by using 2:4-dinitrophenylhydrazine.

Identification of 2:6-*dichlorophenol-indophenol-reducing substance.* The indophenol-reducing substances present in rat urine and produced by rat-liver microsomal enzymes were examined by paper chromatography. Samples of urine and enzyme incubation mixtures were chromatographed on Whatman no. 1 paper that had previously been treated with metaphosphoric acid (1%, w/v) by dipping (Mitchell & Patterson, 1953). The solvent system used was the upper layer of freshly prepared butan-1-ol–acetic acid–water (4:1:5, by vol.; Partridge, 1948). The descending chromatograms were run in the presence of NaCN (Mapson & Partridge, 1949) and, when dry, were sprayed with either a solution of 2:6-dichlorophenol-indophenol or with ammoniacal $AgNO_3$ (Mapson & Partridge, 1949). The only indophenol-reducing substance found had the same R_F as authentic L-ascorbic acid (0·39). Dehydroascorbic acid (R_F 0·48) was also identified in rat urines.

Preparation of microsomal fraction of rat liver. Rat liver from freshly killed animals was homogenized in 4 vol. of ice-cold 0·25 M-sucrose (Schneider, 1948) and the homogenate centrifuged at 900*g* in a refrigerated centrifuge to remove cell debris. The supernatant was recentrifuged at 20 000*g* for 10 min. to remove mitochondria and the microsomes were then aggregated by centrifuging at 90 000*g* for 40 min. in a Spinco model L preparative ultracentrifuge. The microsomes were washed by resuspending them in ice-cold 0·25 M-tris buffer (pH 7·5) followed by centrifuging at 90 000*g* for 40 min. The temperature of the preparations was maintained between 0° and 5° and prepared microsomes were stored for up to 48 hr. at −20°.

Microsomal ascorbic acid synthesis. For ascorbic acid synthesis a typical incubation mixture contained the microsomes from about 0·7 g. of liver resuspended in 0·1 M-sodium phosphate buffer (pH 7·4) containing 0·13 M-sucrose (2·0 ml.) and a freshly prepared solution of L-galactono-γ-lactone (2·5 mg.), 1:1:1-trichloro-2-methylpropan-2-ol (2·0 mg.; Isherwood, Mapson & Chen, 1960) and $Na_4P_2O_7$ (18·0 mg.; Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1960) in 0·1 M-phosphate buffer (pH 7·4) containing 0·25 M-sucrose (6·0 ml.). The mixture was incubated for 1 hr. at 37° (Suzuki, Mano & Shimazono, 1960) with shaking in the presence of air; boiled microsomes were used as a blank. After incubation the enzyme was inactivated by the addition of metaphosphoric acid (12%, w/v; 2·0 ml.) and portions were titrated with 2:6-dichlorophenol-indophenol.

RESULTS AND DISCUSSION

Urinary excretion of ascorbic acid

The normal daily excretion of ascorbic acid by pairs of rats was determined and thereafter for 16 days after a single injection of a compound. Intraperitoneal injection of 1:2-5:6-dibenzanthracene (dibenz[*a*, *h*]anthracene) (50 mg./kg.) increased both the 'total' and 'indophenol-reducing' ascorbic acid excretion of two male rats (Fig. 1). The values obtained by titration with 2:6-dichlorophenol-indophenol were lower but parallel with those for 'total' ascorbic acid; in further experiments titration with 2:6-dichlorophenol-indophenol alone was used to measure ascorbic acid excretion.

The non-carcinogenic hydrocarbons, phenanthrene and anthracene, gave no significant increase in ascorbic acid excretion (Table 1). The 14-fold increase in ascorbic acid excretion produced by 50 mg./kg. of 1:2-5:6-dibenzanthracene was decreased when 20 mg./kg. of 2-diethylaminoethyl 2:2-diphenylvalerate hydrochloride (SKF 525A)

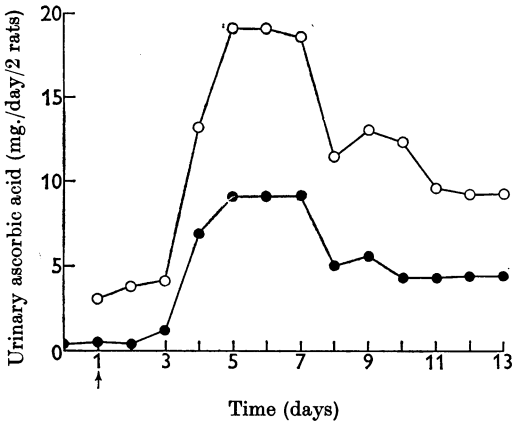

Fig. 1. Urinary ascorbic acid excretion measured by titration with 2:6-dichlorophenol-indophenol (●) and by formation of 2:4-dinitrophenylhydrazone (○) after an intraperitoneal injection (↑) of 1:2-5:6-dibenzanthracene (50 mg./kg.) to two male Wistar rats.

was injected at the same time. This compound (Brodie *et al.* 1955) inhibits the metabolism of drugs by a variety of pathways and it affects particularly microsomal systems, the main site for hepatic ascorbic acid synthesis (Mapson, 1960). 3-Methylcholanthrene gave a smaller increase in ascorbic acid excretion than has been reported by Conney *et al.* (1959). Inscoe & Axelrod (1960) found that treatment of rats with 3:4-benzopyrene (benzo[*a*]-pyrene) increased microsomal glucuronide synthesis *in vitro* by 40% in males and over 250% in females.

The liver carcinogens 4-dimethylaminoazobenzene and 3′-methyl-4-dimethylaminoazobenzene increased excretion of ascorbic acid as did injection of the non-carcinogenic isomeric 2-methyl-4-dimethylaminoazobenzene and 4′-methyl-4-dimethylaminoazobenzene (Table 1). Dimethylnitrosamine, which Magee & Barnes (1956) have shown to produce hepatic tumours in rats, was without effect on ascorbic acid excretion, whereas bromobenzene and to a lesser extent carbon tetrachloride, both of which produce liver necrosis (Popper, de la Huerga & Koch-Weser, 1954; Lamson & Wing, 1926), increased ascorbic acid excretion. Thioacetamide and ethionine, which produce toxic liver injury (Gupta, 1956; Popper *et al.* 1954) and are also carcinogenic (Fitzhugh & Nelson, 1948; Popper, de la Huerga & Yesinick, 1953), increased ascorbic acid excretion.

The injection of a solution of tannic acid, which Korpassy (1959) has shown to have both a necrotic and a hepatocarcinogenic action, gave no increase in ascorbic acid excretion, nor did two tannic acid-containing extracts, quebracho and mimosa, which Kirby (1960) has found to cause liver cancer.

The effects on urinary ascorbic acid concentrations of injections of lead acetate, which causes kidney tumours (Zollinger, 1953), were not conclusive (Table 1) but some delayed increase in excretion was apparent. Treatment with 4-amino-4′-fluorobiphenyl, which also causes kidney tumours (Matthews & Walpole, 1958), gave an immediate increase in ascorbic acid excretion. A more marked increase was produced by a similar dose of 4-aminobiphenyl, which Melick, Escue, Naryka, Mezera & Wheeler (1955) have found to cause cancer of the bladder in man. Although dextran C, which does not induce cancer on injection into rats, gave an increase in urinary ascorbic acid, the iron–dextran complex Imferon, which Richmond (1959) found to cause cancer at the site of injection, had no effect on ascorbic acid excretion. 2-Acetamidofluorene has, amongst other carcinogenic properties, been reported to produce tumours of the urinary tract in the Slonaker strain of rats but not in Wistar rats (Wilson, De Eds & Cox, 1941; Bielschowsky, 1944). The urinary ascorbic acid excretion level was increased in both of these two strains, but the increase was greater in the Wistar rats (Table 1). Injection of sodium arsenite was without effect on urinary ascorbic acid.

The enhanced excretion of ascorbic acid produced by injection of the hypnotic 2-*iso*propyl-4-pentenoyl)urea (Sedormid) and the non-hypnotic allyl-*iso*propylacetamide (Table 1) is similar to that found to occur with other narcotic compounds (Longenecker *et al.* 1940; Burns *et al.* 1960). In addition to producing liver necrosis Sedormid and allyl*iso*propylacetamide cause porphyrinuria (Goldberg & Rimington, 1954). The injection of Tween 80 gave a delayed increase in urinary ascorbic acid excretion. Nitrogen mustard produced variable responses but neither croton oil nor chlorambucil increased urinary ascorbic acid excretion.

Ascorbic acid biosynthesis by rat-liver microsomes

The increased urinary excretion of ascorbic acid which occurs after treatment of rats with a wide variety of compounds has been attributed to an increase in microsomal ascorbic acid synthesis by the glucuronic acid pathway (Burns *et al.* 1960). After establishing normal values for the microsomal synthesis and the urinary excretion of ascorbic acid in a group of male rats, 12 were injected with 1:2-5:6-dibenzanthracene (50 mg./kg.) which has been shown (Table 1) to increase ascorbic acid excretion. The daily urinary excretion of one pair of rats was followed; the others were killed in pairs at intervals after injection. Microsomes were prepared from the homogenized livers and the level of microsomal ascorbic acid synthesis was determined.

Table 1. *Effect of foreign compounds on urinary ascorbic acid excretion in the rat*

Except where stated, male Wistar rats (200 g.) were used and compounds were administered intraperitoneally. Figures in parentheses refer to the day after the injection on which the highest value was recorded.

Compound	Dose (mg./kg.)	Mean ascorbic acid excretion (mg./day/2 rats)		Highest values (mg./day/2 rats)	
		Before injection (8 days)	After injection (16 days)	Before injection	After injection
1:2-5:6-Dibenzanthracene	1·0	1·04	0·67	1·90	1·44 (9)
	2·0	0·69	0·69	1·40	2·08 (9)
	5·0	0·47	0·38	1·14	0·74 (11)
	5·0	0·26	0·51	0·81	1·43 (9)
	5·0*	0·22	0·24	0·51	0·72 (4)
	10·0	0·30	0·49	0·57	1·36 (5)
	10·0†	0·97	2·12	1·59	6·24 (8)
	20	0·43	0·68	1·25	1·97 (7)
	50	0·69	4·96	1·18	10·00 (2)
	50‡	0·45	2·68	0·69	8·00 (10)
	50§	0·76	2·43	1·47	4·50 (2)
1:2-5:6-Dibenzanthracene	50	—	—	—	—
SKF 525A	20	0·71	2·31	1·66	4·00 (11)
Anthracene	100	0·60	0·52	1·00	1·12 (9)
Phenanthrene	100	0·26	0·35	0·35	1·18 (4)
3-Methylcholanthrene	20	0·53	1·29	0·96	2·19 (6)
4-Dimethylaminoazobenzene	100	1·24	1·49	1·56	3·70 (8)
3′-Methyl-4-dimethylaminoazobenzene	100	0·99	1·48	1·70	3·84 (16)
2-Methyl-4-dimethylaminoazobenzene	100	0·71	1·25	1·26	2·08 (10)
4′-Methyl-4-dimethylaminoazobenzene	100	0·44	1·12	0·71	2·86 (9)
Dimethylnitrosamine	15	0·25	0·29	0·50	0·92 (5)
Bromobenzene	500	0·85	0·90	1·50	6·25 (1)
Carbon tetrachloride	1·0‖	0·44	0·58	0·55	1·70 (1)
Thioacetamide	100	0·92	1·01	1·85	5·26 (1)
Ethionine	250	0·23	0·41	0·28	1·11 (2)
Tannic acid	100	0·29	0·44	0·61	1·00 (12)
Quebracho extract	100	0·57	0·79	0·98	1·52 (15)
Mimosa extract	100	0·68	0·60	0·91	1·12 (7)
Lead acetate	50	0·88	0·80	1·43	2·56 (8)
	50	1·36	2·38	1·82	5·27 (7)
4-Amino-4′-fluorobiphenyl	100	0·79	0·64	1·25	2·18 (1)
4-Aminobiphenyl	100	0·89	1·13	1·47	4·17 (1)
Dextran C	5·0‖	0·30	1·15	0·42	2·44 (12)
Imferon	2·5‖	0·21	0·77	0·42	1·67 (10)
	2·5‖	0·32	0·40	0·56	1·04 (15)
	5·0‖	1·22	0·93	1·96	2·34 (3)
	10·0‖	1·26	0·37	1·89	0·72 (7)
2-Acetamidofluorene	100	0·59	1·17	0·93	2·70 (4)
	100†	0·43	0·53	0·67	1·47 (1)
Sodium arsenite	5·0	1·23	0·41	1·92	0·86 (2)
Sedormid	100	0·19	0·67	0·24	2·38 (1)
Allyl*iso*propylacetamide	100	1·00	0·99	1·29	5·55 (1)
Croton oil	25	0·38	0·43	1·00	0·74 (6)
Methylbis(2-chloroethyl)amine	0·5	0·25	0·27	0·29	0·57 (16)
	1·0	0·35	0·38	1·35	1·43 (1)
	1·0	0·39	1·09	0·52	2·34 (16)
Chlorambucil	10·0	0·49	0·60	1·21	1·05 (7)
Tween 80	2·5†	0·67	0·76	1·15	2·04 (12)
	5·0†	1·18	1·23	1·67	3·85 (9)

* Administered subcutaneously. † Male Slonaker rats (200 g.). ‡ Female Wistar rats (200 g.).
§ Male Wistar rats (50 g.). ‖ ml./kg.

Whilst an eightfold increase in urinary ascorbic acid had occurred by the fifth day after injection, only a small increase in microsomal ascorbic acid synthesis was detected.

After 1:2-5:6-dibenzanthracene treatment urinary ascorbic acid excretion by a pair of male rats rose from an average of 1·25 mg./day before treatment to a mean figure of 4·37 mg./day for the 15 days after the injection (Fig. 2). Burns, Mosbach & Shulenberg (1954) have found, using L-[1-^{14}C]ascorbic acid, that the turnover rate for ascorbic acid was 10·7 mg./100 g. body wt. in

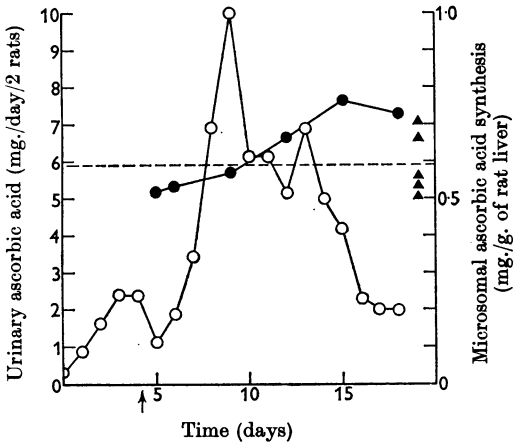

Fig. 2. Effect of an intraperitoneal injection of 1:2-5:6-dibenzanthracene (50 mg./kg.) (↑) on the microsomal synthesis *in vitro* from L-galactono-γ-lactone (●) and the urinary excretion (○) of ascorbic acid by male rats. The level of ascorbic acid synthesis by liver microsomes from pairs of normal rats is shown (▲) (mean for 10 rats – – –). Ascorbic acid was determined by titration with 2:6-dichlorophenol-indophenol.

normal rats and that this was increased in Chloretone-treated rats to 21·5 mg./100 g. body wt. In the present studies on microsomes *in vitro* 0·6 mg. of ascorbic acid/g. of liver/hr. was synthesized from L-galactono-γ-lactone, which is equivalent to a production of 86 mg./100 g. body wt. The high rate of ascorbic acid synthesis *in vitro* suggests that the speed of conversion of L-galactono-γ-lactone into ascorbic acid was not a limiting factor in ascorbic acid biosynthesis *in vivo*. This could explain why only a small increase in microsomal ascorbic acid formation was found after dibenzanthracene treatment.

The drug-induced increase in ascorbic acid formation is apparently brought about by some change other than an increase in the activity of the microsomal enzyme which converts L-galactono-γ-lactone into ascorbic acid. This agrees with the finding of Burns *et al.* (1960) that pretreatment with a compound which increases ascorbic acid excretion results in an increase in the metabolism of glucose through the glucuronic acid pathway, the ascorbic acid being formed from the further metabolism of glucuronic acid. Kanda & Sakamoto (1960) reported that although an increased excretion of ascorbic acid occurs in alloxan-diabetic rats, ascorbic acid breakdown by a hepatic system *in vitro* is decreased. The decrease, which is abolished by insulin treatment, does not occur in barbital-treated rats. Isherwood *et al.* (1960) have shown that the addition of chloral hydrate to rat-liver-microsome preparations increases the rate of ascorbic acid synthesis from L-gulono-γ-lactone by inhibiting the enzymic hydrolysis of this lactone. The rate of ascorbic acid synthesis *in vivo* may be controlled in part by regulation of the amount of substrate available for conversion into ascorbic acid, and some of the varied compounds that accelerate ascorbic acid synthesis in the rat may act by inhibiting enzymic hydrolysis of L-gulono-γ-lactone.

SUMMARY

1. Some foreign compounds increase synthesis and excretion of ascorbic acid in rats.

2. A new method for the preparation of L-galactono-γ-lactone by high-pressure catalytic reduction of D-galacturonic acid is described. The rate of conversion of L-galactono-γ-lactone into ascorbic acid was the same in microsomes from normal rats and rats treated with 1:2-5:6-dibenzanthracene, which caused increased ascorbic acid excretion in the whole animal.

This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council, the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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Studies on Incorporation of Amino Acids into Protein in Isolated Rat-Liver Ribosomes

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(*Received* 28 *March* 1961)

Cells contain microsomal particles, or ribosomes (Roberts, 1958), 10–25 mμ in diameter, which contain ribonucleoprotein on which, it is generally believed, amino acids, activated by reactions occurring in the cell sap (Hoagland, Keller & Zamecnik, 1956; Hoagland, Zamecnik & Stephenson, 1957), are assembled into polypeptide chains during the biosynthesis of proteins (see Hoagland 1960, for review). This view is greatly strengthened by the results obtained by Littlefield, Keller, Gross & Zamecnik (1955) in experiments with whole animals, which are consistent with the hypothesis that proteins are formed in the ribosomes and are transferred, via the lipoprotein of the endoplasmic reticulum, to the soluble fraction of the cell.

In the experiments of Littlefield *et al.* (1955) ribosomes were prepared from microsomes by treatment of the latter with sodium deoxycholate (Palade & Siekevitz, 1956), which solubilizes the lipoprotein part of the microsomes (Strittmatter & Ball, 1952). The ribosomes were reported (Littlefield & Keller, 1957) to be incapable of incorporating radioactive amino acids into protein *in vitro* in a system of the type described by Zamecnik & Keller (1954). The implication was that treatment with deoxycholate may have damaged the ribosomes.

Ribosomes from many tissues have been intensively studied recently and their ability to aggregate and disperse in a manner dependent on the pH and composition of the suspending medium and in particular on the concentration of magnesium ions in the medium has excited much attention (Tissière & Watson, 1958; Gillchriest & Bock, 1958; Wagman & Trawick, 1958; Bolton, Hoyer & Ritter, 1958; Petermann, Hamilton, Balis, Samarth & Pecora, 1958; Tissière, Watson, Schlessinger & Hollingsworth, 1959; Huxley & Zubay, 1960; Petermann & Hamilton, 1961). In view of these reports it was thought that deoxycholate could be used to prepare ribosomes from rat liver in a form capable of incorporating amino acids into protein *in vitro*, if care were taken to use the optimum concentration of magnesium ions during the preparation. It was soon found that ribosomes could be prepared from rat liver by the normal deoxycholate method, in the absence of magnesium, and that they would incorporate amino acids into protein *in vitro*. The result has been briefly reported (Korner, 1959*a*, 1960*a*), and this paper records the methods used to prepare the ribosomes, reports on their properties and gives the results of some studies carried out with them. Since the earlier