TEST FOR MUTAGENICITY

in BACTERIA STRAINS in the ABSENCE and PRESENCE OF A LIVER PREPARATION

The substance ..M.03.-.77..(.=.OCTOPIROX).....
has been investigated with the mutagenicity test originally described by Bruce N. AMES (see references 1 - 7).
A lot of organic chemicals causing cancer are reactive chemical species or they must be converted to such by metabolic processes. They will readily react with nucleophile If reactive metabolites are generated within mammalian cells they will attack among other macromolecules, the nucleic acid Such reactions may form the basic steps in cancer induction. For production of reactive chemical species can be detected biologically by studying the mutations, which are induced in bacteria consequent on the chemicals reaction with the hereditary material (DNA). Therefore bacteria are used as Salmonella typhimurium strains which have been specifically developed for detecting reactive metabolites. For their growth these strains require the addition of histidine. If they are treated with agents reacting with the bacterial DNA a certain number of bacteria in the population will be mutate so that they no longer require the amino acid for growth. Such mutants will grow (on an agar plate) as single colonies on a background lawn of non-mutated bacteria. The number of colonies can be counted, agents which cause mutation will increase the number of colonies per agar plate. In any bacterial population there will be a certain number of spontaneous mutants and this will differ from strain to strain. For TA 98^{+} and TA 100^{++} the spontaneous mutation frequency is usually higher than for TA 1535^{++} and TA 1537^{+}.

$^{+}$ (strain with frameshift mutation)
$^{++}$(strain with base-pair substitution)

The first two strains are more sensitive to mutagens than the latter.
Different bacterial strains are used because each strain responds to only a particular class of chemical. A chemical which is mutagenic for one strain is not necessarily mutagenic for the other three. For better understanding the genotype of the tester strains is demonstrated in the following table.

Genotype of the TA strains used for Mutagen Testing+

Histidine mutation		Additional mutations		
hisG46	hisD3052	LPS	Repair	R fact...
TA1535	TA1537	rfa	⊿ uvrB	-
TA100	TA98	rfa	⊿ uvrB	+R

+All strains were originally derived from S. typhimurium LT_2. The deletion (⊿) through uvr B also includes the nitrate reductase (chl) and biotin (bio) genes.

MATERIAL AND METHODS

The methods used are published in detail by AMES et al. (see reference 2). In brief the procedure is the following one:
The tester strains TA 98, 100, 1535, and 1537 are maintained at-60° C and recultivated for the test in fresh nutrition brot
The S-9 fraction is prepared from the microsomes of a liver homogenate as described by AMES also. Sprague-Dawley rats are injected once with 500 mg/kg of polychlorinated biphenyl (PCB) = Aroclor 1254 (Monsanto, St. Louis, Mo. USA) in corn oil (200 mg/ml) i.p. five days before killing the animals.

The livers are prepared in cooled sterile glass ware using cooled solutions by homogenizing the livers in a Potter Elvehjem Apparatus. This homogenate is centrifuged by 9000 g for 10 minutes (Sorvall RC2-B, in SS-34 head 8700 rev/min.). 1 ml of the supernatant contains microsomes of around 250 mg of liver. This preparation is the S-9 fraction which is stored in deep frozen stage.
Samples of it are thawed immediately before each test and the S-9 mix is prepared. It contains per ml:

S-9 fraction	(0.04 - 0.1 ml)
$MgCl_2$	(8 µmoles)
KCl	(33 µmoles)
Glucose-6-phosphat	(5 µmoles)
NADP	(4 µmoles)
Natriumbosphat, pH 7,4	(100 µmoles)

The exact dosage of every charge of S-9 fraction is evaluated in pretests with known negative and positive substances. The tester strains are checked for their reactivity also. Sterilit controls are also routinely performed in each experiment.
All dilutions of the substances in test are prepared with Dimethylsulfoxid (Schwarz-Mann, spectrophotometric grade), or with different dilutants such as ethanol or Aq. dest. This is reported in each special case in the protocols.

The dosages range from 0.2 to 500 γ , and the tests are performed without and with activation with S-9. The bacterial plates are incubated for 48 hours at 37° C and then the numbers of reverted bacterial colonies counted. Control plates for determining the spontaneous mutation rate are investigated in the same way.
The results are listed in the attached figures.

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tested with 4 bacterial strains showed N O mutagenicity neither in the presence nor absence of rat liver preparation in the dosage range of 500 y to 0,2 y.

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$y = \gamma = 10^{-6}$ g/ml = ppm = µg/ml

REFERENCES

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20.7.1977

AMES-Test..... Protocol-No. 52/77

Preparation in test M 03 - 77

Microsome fraction S9 Rat C1 Induction by Aroclor 1254

Tester strains: mutants of S.typhimurium TA 98

Dilutant: H_2O

Time of incubation 48 hrs. 37° C

Tester strains TA 98	Preparation in test mutants/petri dish 0.1 ml	Spontaneus mutants petri dish 0.1 ml	Evaluation
500 y	Ø - Ø	12 - 19 - 19	
500 y + S9	Ø - Ø	14 - 14 - 16	
250 y	Ø - Ø	12 - 19 - 19	
250 y + S9	Ø - Ø	14 - 14 - 16	
100 y	4 - 5	12 - 19 - 19	
100 y + S9	10 - 11	14 - 14 - 16	
20 y	5 - 8	12 - 19 - 19	
20 y + S9	13 - 15	14 - 14 - 16	
2 y	10 - 11	12 - 19 - 19	
2 y + S9	15 - 15	14 - 14 - 16	
Ø = tested, but no bacterial growth			

AMES-Test 20.7.1977 Protocol-No. 52/77

Preparation in test M 03 - 77

Microsome fraction S9 Rat C Induction by Aroclor 1254

Tester strains: mutants of S.typhimurium ~~TA XXXX98~~ TA 100 ~~TA XX535~~ ~~TA XX537~~

Dilutant: H_2O

Time of incubation 48 hrs. 37° C

Tester strains TA 100	Preparation in test mutants/petri dish 0.1 ml	Spontaneous mutants petri dish 0.1 ml	Evaluation
500 y	Ø - Ø	65 - 70 - 71	
500 y + S9	Ø - Ø	62 - 74 - 77	
250 y	Ø - Ø	65 - 70 - 71	
250 y + S9	Ø - Ø	62 - 74 - 77	
100 y	32 - 33	65 - 70 - 71	
100 y + S9	33 - 35	62 - 74 - 77	
20 y	44 - 58	65 - 70 - 71	
20 y + S9	58 - 57	62 - 74 - 77	
2 y	54 - 70	65 - 70 - 71	
2 y + S9	44 - 45	62 - 74 - 77	

Ø = tested, but no bacterial growth

AMES-Test 20.7.1977 Protocol-No. 52/77

Preparation in test M 03 - 77

Microsome fraction S9 Rat ♂ Induction by Aroclor 1254

Tester strains: mutants of S.typhimurium ~~TA XXXX~~ ~~TA XXXX~~ TA 1535 ~~TA XXXX~~

Dilutant: H_2O

Time of incubation 48 hrs 37° C

Tester strains TA 1535	Preparation in test mutants/petri dish 0.1 ml	Spontaneous mutants petri dish 0.1 ml	Evaluation
500 y	Ø - Ø	4 - 9 - 13	
500 y + S9	Ø - Ø	8 - 9 - 18	
250 y	Ø - Ø	4 - 9 - 13	
250 y + S9	Ø - Ø	8 - 9 - 18	
100 y	8 - 14	4 - 9 - 13	
100 y + S9	6 - 7	8 - 9 - 18	
20 y	13 - 19	4 - 9 - 13	
20 y + S9	11 - 14	8 - 9 - 18	
2 y	21 - 24	4 - 9 - 13	
2 y + S9	14 - 19	8 - 9 - 18	
Ø = tested, but no batcterial growth			

AMES-Test 2.8.1977 Protocol-No. 52 a/77

Preparation in test M 03 - 77

Microsome fraction S9 Rat ♂ Induction by Aroclor 1254

Tester strains: mutants of S.typhimurium ~~XXXXXX98~~ TA 100 ~~XXXXX536~~ ~~XXXXX537~~

Dilutant: DMSO

Time of incubation 48 hrs. 37° C

Tester strains TA 100	Preparation in test mutants/petri dish 0.1 ml	Spontaneous mutants petri dish 0.1 ml	Evaluation
20 y	50 - 57	87 - 94 - 101	
20 y + S9	58 - 62	59 - 64 - 66	
2 y	62 - 71	87 - 94 - 101	
2 y + S9	75 - 89	59 - 64 - 66	
0,2 y	68 - 68	87 - 94 - 101	
0,2 y + S9	68 - 79	59 - 64 - 66	

AMES-Test 2.8.1977 Protocol-No. 52 a/77

Preparation in test M 03 - 77

Microsome fraction S9 Rat ♂ Induction by Aroclor 1254

Tester strains: mutants of S.typhimurium TA 98

Dilutant: DMSO

Time of incubation 48 hrs. 37° C

Tester strains TA 98	Preparation in test mutants/petri dish 0.1 ml	Spontaneous mutants petri dish 0.1 ml	Evaluation
20 y	7 - 12	13 - 14 - 20	
20 y + S9	13 - 14	12 - 13 - 21	
2 y	10 - 16	13 - 14 - 20	
2 y + S9	9 - 12	12 - 13 - 21	
0,2 y	8 - 10	13 - 14 - 20	
0,2 y + S9	8 - 16	12 - 13 - 21	

AMES-Test 20.7.1977 Protocol-No. 52/77

Preparation in test M 03 - 77

Microsome fraction S9 Rat ♂ Induction by Aroclor 1254

Tester strains: mutants of S.typhimurium ~~TXXXX88~~ ~~TXXXX100~~ ~~TXXX535~~ TA 1537

Dilutant: H_2O

Time of incubation 48 hrs 37° C

Tester strains TA 1537	Preparation in test mutants/petri dish 0.1 ml	Spontaneous mutants petri dish 0.1 ml	Evaluation
500 y	Ø - Ø	3 - 4 - 5	
500 y + S9	Ø - Ø	2 - 5 - 5	
250 y	Ø - Ø	3 - 4 - 5	
250 y + S9	Ø - Ø	2 - 5 - 5	
100 y	2 - 3	3 - 4 - 5	
100 y + S9	3 - 3	2 - 5 - 5	
20 y	3 - 5	3 - 4 - 5	
20 y + S9	2 - 3	2 - 5 - 5	
2 y	2 - 4	3 - 4 - 5	
2 y + S9	2 - 4	2 - 5 - 5	
Ø = tested, but no bacterial growth			

AMES-Test 2.8.1977 Protocol-No. 52 a/77

Preparation in test M 03 - 77

Microsome fraction S9 Rat ♂ Induction by Aroclor 1254

Tester strains: mutants of S.typhimurium ~~TA XXXX98~~ ~~TA XXXX100~~ TA 1535 ~~TA XXXX97~~

Dilutant: DMSO

Time of incubation 48 hrs. 37° C

Tester strains TA 1535	Preparation in test mutants/petri dish 0.1 ml	Spontaneous mutants petri dish 0.1 ml	Evaluation
20 y	5 - 13	16 - 17 - 17	
20 y + S9	7 - 8	9 - 16 - 17	
2 y	7 - 12	16 - 17 - 17	
2 y + S9	8 - 13	9 - 16 - 17	
0,2 y	8 - 12	16 - 17 - 17	
0,2 y + S9	9 - 17	9 - 16 - 17	

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AMES-Test..... Protocol-No. 52 a/77

Preparation in test M 03 - 77

Microsome fraction S9 Rat ♂ Induction by Aroclor 1254

Tester strains: mutants of S.typhimurium ~~TA XXXX~~ ~~TA XXXX~~ ~~TA XXXX~~ TA 1537

Dilutant: DMSO

Time of incubation 48 hrs 37° C

Tester strains TA 1537	Preparation in test mutants/petri dish 0.1 ml	Spontaneous mutants petri dish 0.1 ml	Evaluation
20 y	2 - 4	2 - 2 - 4	
20 y + S9	2 - 3	3 - 3 - 5	
2 y	4 - 7	2 - 2 - 4	
2 y + S9	1 - 3	3 - 3 - 5	
0,2 y	4 - 5	2 - 2 - 4	
0,2 y + S9	3 - 6	3 - 3 - 5	

Compound	0.1ml/plate	S-9 Mix	Revertant colonies / plate TA98	TA100	TA1535	TA1537
Positive controls						
2-acetylamino-fluorene	10 µg	+	402 415			
Benzo(a)pyrene	5 µg	+		680 730		
ß-Naphthylamine	10 µg	+			275 310	
Neutralred	100 µg	+				309 325
4-Nitro-o-phenylendiamine	50 µg	-	2700 3000	580 642		80 85
Streptozotozin	1 µg				720 766	