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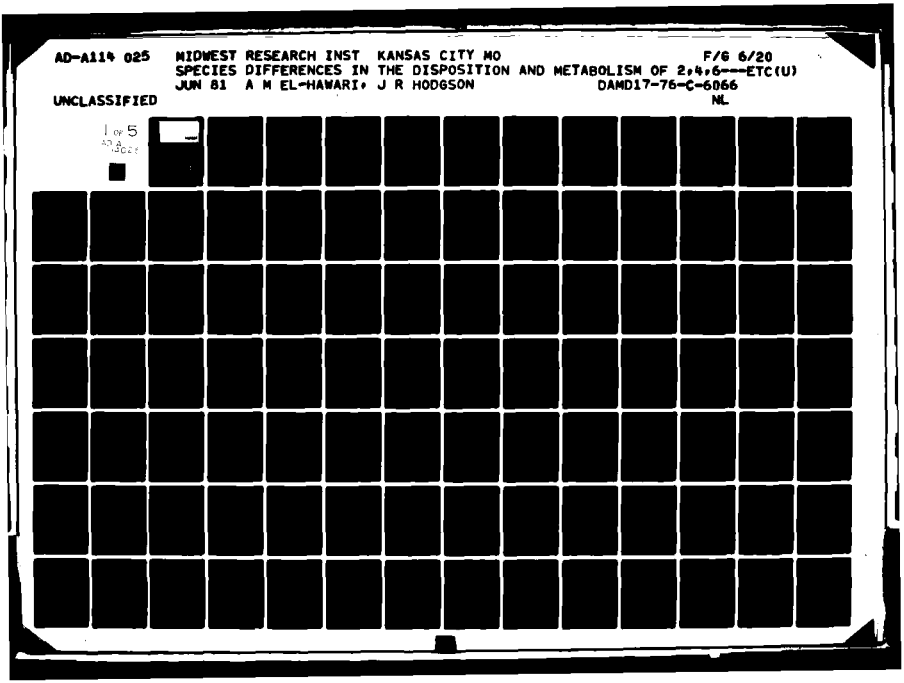

REPORT

MRI Project No. 4274-B

SPECIES DIFFERENCES IN THE DISPOSITION AND METABOLISM OF 2,4,6-TRINITROTOLUENE AS A FUNCTION OF ROUTE OF ADMINISTRATION

FINAL REPORT

A. Monaem El-hawari
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Mary D. Sawyer
Maxine Hainje
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June 1981

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, MD 21701

Contract No. DAMD17-76-C-6066

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Kansas City, Missouri 64110

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2,4,6-Trinitrotoluene	Metabolism	Oral	Rat
TNT	Absorption	Dermal	Dog
Species Differences	Tissue Distribution	Intratracheal	Rabbit
Disposition	Excretion	Mouse	

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The disposition and metabolism of 2,4,6-trinitrotoluene (TNT) was studied in rats, mice, rabbits, and dogs following oral, dermal or intratracheal administration of single doses of ^{14}C-ring labeled compound. The objective was to determine possible species and sex differences as a function of route of administration as a rationale for the design of chronic studies.

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20. Abstract (continued)

TNT was absorbed in all species by all routes of administration with the most extensive absorption occurring after intratracheal instillation. Dermal absorption was highest in rabbits followed by mice, rats, and dogs. Species differences in the rate of oral absorption could not be accurately assessed. Excretion was primarily in urine and to a lesser extent in feces. Extensive biliary excretion was also noted. Blood and tissue levels in females were generally higher than in males.

TNT was extensively metabolized in all species; radioactivity excreted in urine primarily as the glucuronide conjugates. Most metabolites were reduction products including the 2- and 4-hydroxylamine and 2- and 4-monoaminodinitro and 2,6- and 4,6-diaminomononitro derivatives. Trace quantities of TNT, trinitrobenzyl alcohol and trinitrobenzoic acid were detected occasionally.

Urinary metabolic profiles were similar qualitatively in mice, rats, and dogs; profiles in rabbits were dissimilar. Also, metabolic profiles demonstrated after intratracheal instillation differed significantly from those obtained after oral or dermal administration.

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PREFACE

This report was prepared at Midwest Research Institute, 425 Volker Boulevard, Kansas City, Missouri 64110, under U.S. Department of the Army Contract No. DAMD-17-76-C-6066 entitled "Evaluation of Difference in Mammalian Metabolism of Trinitrotoluene (TNT) as a Function of Route of Administration and Carcinogen Testing." The work was supported by the U.S. Army Medical Research and Development Command, Department of the Army. Cpt. Ronald N. Shiotsuka, MSC, Environmental Protection Research Division, U.S. Army Medical Bioengineering Research and Development Laboratory, was the Contract Officer's technical representative.

The work was conducted in the Biological Science Division under the direction of Dr. William B. House, between June 29, 1976 and March 31, 1978, and Dr. Harold M. Hubbard, between April 1 and November 30, 1978. The experimental work was directed by Dr. Cheng-Chun Lee, Deputy Director, with Dr. John R. Hodgson, Head, Biochemical and Developmental Pharmacology, and Dr. A. Monaem El-hawari, Senior Toxicologist, as the successive Principal Investigators. Dr. J. Mark Winston performed and supervised the inhalation investigations. Ms. Mary D. Sawyer and Ms. M. Hainje, Junior Biologists, performed the animal experiments, radioactivity and TLC analysis. Mr. W. B. Butron, Associate Chemist, synthesized the potential metabolites. Dr. E. Murrill, Senior Advisor for Chemistry, supervised the HPLC analysis.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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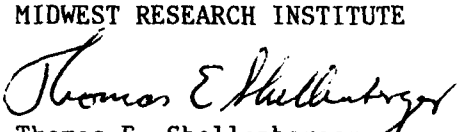

Thomas E. Shellenberger
Director
Toxicology Department

June 1981

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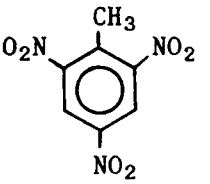
SPECIES DIFFERENCES IN THE DISPOSITION AND METABOLISM OF 2,4,6-TRINITROTOLUENE AS A FUNCTION OF ROUTE OF ADMINISTRATION

EXECUTIVE SUMMARY

The disposition (absorption, tissue distribution, and excretion) and metabolism of 2,4,6-trinitrotoluene (TNT, I) were studied in rats, mice, rabbits, and dogs after oral, dermal, or intratracheal administration of single doses of the ring-^{14}C-labeled compound. The primary objective of these studies was to determine the species differences, if any, in the metabolic fate of TNT as a function of route of administration for possible use as a rationale for selecting an appropriate species, sex, and route of exposure for subsequent chronic studies. Specifically, the intent was to evaluate the metabolic behavior of TNT after oral, inhalation, and dermal exposures in order to establish if oral exposure could be used in lieu of other routes in any subsequent carcinogenicity studies. Since TNT aerosols prepared using methods reported herein were not adequate for inhalation exposure, the intratracheal instillation method was used in an attempt to simulate pulmonary absorption of the test chemical.



(I) TNT

TNT administered orally to rats, mice, rabbits, and dogs was readily absorbed and excreted mainly in urine and to a lesser extent in feces. Major portions of the administered doses were recovered in the GI tracts (Table A). Urine of rats and mice, but not of rabbits and dogs, was bright red in color. The extent of absorption could not be accurately assessed from these studies since radioactivity recovered in the feces and GI tracts represents a balance between absorption, biliary excretion, and intestinal reabsorption. At 24 hr, blood and tissue of dogs contained higher radioactivity (percent of dose) than did blood and tissue of rats, mice, and rabbits. Generally, higher ^{14}C levels were recovered in blood and tissue of female animals. Blood, liver, kidneys, and occasionally spleen and lungs contained high levels of radioactivity; rabbit lung tissue contained 9 to 14 times higher ^{14}C levels than did blood. Other tissues, including brain and muscle, contained detectable levels of radioactivity.

TABLE A

RECOVERY OF RADIOACTIVITY (PERCENT OF DOSE) AT 24 HR AFTER ORAL ADMINISTRATION OF ^{14}C-TNT

	Rats		Mice		Rabbits		Dogs	
	Male	Female	Male	Female	Male	Female	Male	Female
Urine	52.72	64.55	41.91	42.87	66.30	78.86	55.92	60.16
Feces	8.05	2.06	22.01	8.96	1.78	1.83	5.41	16.80
GI Tract	29.76	33.94	13.45	7.42	7.50	4.72	10.00	4.40
Blood	0.20	0.29	0.90	0.07	0.28	0.44	1.38	1.96
Tissue	0.89	1.59	2.18	1.11	1.80	3.10	4.64	4.96
Recovery	91.62	102.43	80.06	60.44	77.65	88.94	77.35	88.26

Following dermal application, TNT was absorbed by the four species studied. Absorption was highest in rabbits followed by mice, rats, and dogs (Table B). Most of the TNT absorbed was eliminated in urine. Radioactivity was also recovered in the feces and GI tracts indicating probable excretion via bile. Total urinary and fecal excretion at 24 hr following dermal application was less than after oral administration of the same dose. As with the oral dosing, urine of dermally dosed rats and mice was bright red. Residual radioactivity was higher in fat of all species following dermal application than after oral dosing. Radioactivity was also highly concentrated in residual bile and liver after both dermal and oral exposure. In rabbits and dogs, absorption and excretion of TNT appeared similar at both dose levels studied (5 and 50 mg/kg) although in dogs, blood content (percent of dose) was higher after the high dose of TNT.

TABLE B

RECOVERY OF RADIOACTIVITY (PERCENT OF DOSE) AT 24 HR AFTER ORAL OR DERMAL TREATMENT WITH ^{14}C-TNT

	Rats		Mice		Rabbits		Dogs	
	Oral	Dermal	Oral	Dermal	Oral	Dermal	Oral	Dermal
Urine	59.54	17.35	59.05	22.68	68.07	52.85	70.50	11.73
Feces	10.72	1.32	24.07	14.17	5.45	7.80	9.00	1.71
GI Tract	20.24	3.11	10.19	3.61	19.74	5.76	14.63	1.68
Blood	0.25	0.23	0.17	0.17	0.40	0.26	1.11	0.26
Tissue	1.44	0.68	0.91	1.04	1.91	1.59	4.15	1.42
Recovery	92.19	22.76	94.39	41.69	95.57	68.26	99.39	16.81

Extensive absorption was demonstrated when a suspension of ^{14}C-TNT was instilled intratracheally into rats. Radioactivity appeared in the blood quickly and decreased slowly during a 4-hr period. Blood ^{14}C levels were higher and the urinary excretion levels were greater in these rats than in rats treated orally under the same conditions (Table C). In bile duct-cannulated rats, large amounts of radioactivity were excreted in the bile, urine, and the GI tract. Urinary and biliary excretion rates were also higher in these rats than in rats treated orally. Enterohepatic circulation of TNT and its metabolites (excretion in bile followed by absorption and reexcretion in urine or feces) seemed to occur. The urine of rats from both routes of administration was bright red. At 4 hr, residual radioactivity in most tissues was higher after intratracheal instillation than after oral administration. After both routes of administration, fat contained the highest content of radioactivity, and lung tissue had higher ^{14}C concentrations than did blood or liver. Levels of ^{14}C in blood and tissues of female rats were about two times higher than in the males.

TABLE C

RECOVERY OF RADIOACTIVITY (PERCENT OF DOSE) AT 4 HR AFTER ORAL OR INTRATRACHEAL ADMINISTRATION OF ^{14}C-TNT

	Intact Rats				Bile Duct-Cannulated Rats			
	Oral		Intratracheal		Oral		Intratracheal	
	Male	Female	Male	Female	Male	Female	Male	Female
Urine	14.63	10.01	19.32	13.23	10.73	8.42	17.50	12.68
Bile	-	-	-	-	11.57	9.67	19.75	14.51
GI Tract	73.70	79.02	18.24	12.06	68.29	64.22	1.79	2.92
Blood	1.34	2.78	2.24	4.29	1.34	2.78	2.24	4.29
Tissue	3.60	6.12	5.80	10.58	3.60	6.12	5.80	10.58
Recovery	93.27	97.93	45.60	40.16	95.53	91.21	47.06	44.98

Because of the presence of four functional groups on the TNT molecule, a variety of metabolites resulting from oxidation, reduction, and conjugation could be formed. Simultaneous oxidation and reduction followed by conjugation is also a possibility. Most TNT metabolic products in urine and bile are highly polar with very low extractability in organic solvents (ether and ethyl acetate). Mild acidification (dilute HCl) before ethyl acetate extraction proved essential for increased recovery. A method was developed for the fractionation of the radioactive urinary metabolites into subgroups according to their solubilities in ether under different pH conditions. Metabolites were separated by thin-layer chromatography (TLC). The use of gas-liquid chromatography (GLC) and high performance liquid chromatography (HPLC) was discontinued after it was apparent that neither technique offered added advantages in the separation of TNT metabolites. Tentative identification of metabolites was carried out by comparing solubility characteristics, reactions with specific spraying reagents, and R_f values of the metabolites with those of standard reference compounds.

TNT was metabolized extensively in all species examined, whether treatment was oral, dermal, or intratracheal. Large portions of the products were conjugated with glucuronic acid, but no conjugation with sulfuric acid was detected. Other conjugates or inorganic salts of TNT metabolites were probably present. Most of the metabolic products were reduction derivatives, including the 2- and 4-hydroxylamines, the 2- and 4-monoaminodinitro and the 2,6- and 4,6-diaminomononitro derivatives. The trinitrobenzyl alcohol and the trinitrobenzoic acid seemed to be present, but confirmation was not possible. The parent compound, TNT, was demonstrated in the urine of some species in only minute quantities. The mild extraction procedures used minimized the alterations of the hydroxylamines to the azoxytoluene, but some of the latter was present, especially after fractionation of the urinary products in the presence of NaOH. Other products of TNT metabolism remain unidentified.

The metabolic profiles of urine from rats, mice, and dogs differed only quantitatively. Urine of rats contained larger amounts of the 4,6-diamine and, to a lesser extent, the 2,6-diamine and either or both of the 2- or 6-monoamines. The 2- and 4-hydroxylamines and some azoxytoluene (probably formed during fractionation) were present in small quantities. The presence of appreciable amounts of the trinitrobenzyl alcohol and trinitrobenzoic acid was suggested by comparison with authentic samples. Metabolic profiles of urine from male and female rats showed no significant differences. The amounts of glucuronides in urine collected from bile duct-cannulated rats were lower than those collected from noncannulated rats. In addition, the 4-hr urine contained more of the polar metabolites and more parent TNT.

Compared to rat urine, mouse urine contained smaller quantities of the polar metabolites and the diamines and more of the monoamines and hydroxylamines. Mouse urine also contained considerable amounts of the trinitrobenzyl alcohol and trinitrobenzoic acid. The metabolic profiles of dog urine contained appreciable amounts of diamines and monoamines and probably the trinitrobenzyl alcohol and the trinitrobenzoic acid. Only traces of the 4-hydroxylamine, the 2-hydroxylamine, and some azoxytoluene (which seemed to be formed during fractionation) were present. Rabbit urine showed an unique profile which differed quantitatively, and probably qualitatively, from that of rats, mice, and dogs. The presence of larger quantities of monoamines and hydroxylamines was demonstrated. In addition, it contained either or both of the diamines, trinitrobenzyl alcohol, and trinitrobenzoic acid. TNT and the azoxytoluene were absent from fresh urine, but some of the latter was formed during fractionation in the presence of NaOH.

Major quantitative differences were demonstrated in the urinary metabolic profiles of orally versus intratracheally treated rats. On the other hand, the differences between urine profiles obtained from orally and dermally treated animals were minimal, although larger amounts of TNT were eliminated after dermal application. The extractable radioactivity increased considerably after β-glucuronidase hydrolysis of urine from different species following different routes of administration. However, major changes in the metabolic profiles were not apparent.

I. INTRODUCTION

Under Contract No. DAMD-17-76-C-6066, entitled "Evaluation of Differences in Mammalian Metabolism of Trinitrotoluene (TNT) as a Function of Route of Administration and Carcinogenic Testing," MRI conducted experimental studies to achieve the following objectives:

1. Develop a suitable method for the generation of an aerosol of TNT in sufficient concentrations for metabolic studies.

2. Determine the disposition (absorption, tissue distribution and excretion) of TNT in four animal species (rat, mouse, rabbit, and dog) after oral, dermal, and intratracheal administration.

3. Develop suitable methods for the characterization of the metabolic products of TNT in the urine of these species.

The primary objective of these studies was to develop a data base for selecting an appropriate animal model for subsequent chronic studies and to determine whether oral administration could be used as an alternative to other (e.g., dermal and inhalation) routes in any future carcinogenicity studies.

The initial approach was to compare the absorption, distribution, metabolism, and elimination of TNT in several species following oral and inhalation exposures. Initial efforts were directed to procurement or synthesis of some potential metabolites, development of methods to separate and identify these metabolites, the conduct of oral dosing studies, and the evaluation of methods to produce aerosols applicable for inhalation exposures. Efforts to generate satisfactory TNT aerosols in concentrations which are suitable for metabolic studies were not successful; and following discussions with the project officer, intratracheal instillation to simulate inhalation exposure was substituted. Dermal exposure studies were subsequently incorporated into the project. The research efforts thereafter were directed to disposition and metabolic studies following oral, dermal, or intratracheal administration of TNT using rats, mice, rabbits, and dogs.

II. BACKGROUND

A. Production and Use

2,4,6-Trinitrotoluene (TNT) was first synthesized by Wilbrand in 1863, but it was not prepared on an industrial scale until 1891. A few years later, it found wide application as an explosive for shells, bombs, and grenades.[1] Millions of tons of TNT were produced during World Wars I and II. In 1973, an estimated 200,000 tons were manufactured in the U.S. Army ammunition plants.[2] TNT is the most widely used military explosive because of its low melting point, comparative safety during manufacture, and stability during transporation and storage.[3,4] It is also used as an intermediate in the synthesis of dyes and photographic chemicals.

B. Human Toxicity

The manufacture of TNT creates fumes of TNT and other decomposition products. Some workers exposed to TNT by breathing the fumes or by skin contact have experienced harmful effects, including liver malfunction[5-7] and decreased ability of the bone marrow to produce blood cells.[8-12] TNT also damages the heart,[13] blood vessels,[14] kidney,[15,16] and pancreas,[17] and probably causes cataracts.[18-23] Exposure to TNT decreases the oxygen-carrying capability of the red blood cells due to formation of methemoglobin[24] and nitric oxide hemoglobin.[25] Hemolytic anemia has been reported in TNT workers deficient in glucose-6-phosphate dehydrogenase.[26,27] Persons poisoned with TNT have urine that is red but not bloody.[28] Deaths have been mainly attributed to jaundice, aplastic anemia, or both.[5,6] To date, no carcinogenic effect has been reported among munitions workers exposed to TNT.[29]

During World War I, a large number of cases of toxic jaundice were reported among TNT workers in the United States and Europe, many of which ended in fatality.[30-33] The implementation of strict hygiene practices during World War II resulted in a dramatic decrease in the number of fatalities. Currently, the Occupational Safety and Health Administration limits exposure to TNT to 1.5 mg/m^3 in air (8-hr time-weighted average). To provide greater protection to munition workers, the U.S. Army has lowered its acceptable TNT exposure levels to 0.5 mg/m^3 over the same time period.

C. Animal Toxicity

Liver and blood diseases have appeared in experimental animals exposed to TNT.[34-39] No pulmonary lesions or lung neoplastic effects have been demonstrated in guinea pigs, rats, or mice exposed to TNT.[29] However, after 6 months of topical application of TNT to Wistar rats, bone marrow cells exhibited chromatid changes, chromosomal breaks, and dislocations, but no change in chromosomal numbers.[40] Studies on TNT mutagenicity using histidine-requiring strains of Salmonella typhimurium (Ames test system) have indicated that TNT is mutagenic. However, the major microbial metabolites of TNT appear to be nonmutagenic.[41]

Experimental animals differ in susceptibility to TNT toxicity. Cats are more sensitive than rats, rabbits, dogs, and monkeys. It has been suggested that these differences are due, at least in part, to differences in the fate of TNT in these species.[42] It has also been shown that various microorganisms biodegrade TNT; Escherichia coli can reduce the nitro groups to the respective amines.[43-45] Degradation of TNT by bacteria and sunlight gives TNT wastewater a pink or red color.

D. Absorption

TNT may enter the body through the gastrointestinal tract, the skin, or the lungs.[46-48] It is believed that the skin is the chief route of absorption.[46] Voegtlin et al.[47] have demonstrated that in humans, skin absorption takes place readily through the hands, neck, and face; oily skin and sweat favors absorption.[49] Although some experiments have demonstrated that TNT is absorbed when introduced as dust in the lower air passages, Putnam and Herman[46] suggested that intoxication via the respiratory tract rarely, if ever, occurs.

During exposure to TNT, the powder may be ingested by mouth and gain access to the stomach. TNT workers have complained about a bitter taste in their mouth. When two human subjects received daily doses of TNT for four successive days, a portion of the TNT administered was recovered from the urine in the form of the reduced metabolite, 2,6-dinitro-4-aminotoluene.[50] Experimentally, guinea pigs fed oral doses of TNT with milk developed diarrhea, and poisoning symptoms were apparent for 3 to 14 days.[51]

TNT was absorbed through the skin of swine as indicated by the presence of the reduced metabolite, 2,6-dinitro-4-aminotoluene in urine.[52] Also, Haythorn[53] reported that guinea pigs and rabbits rubbed repeatedly with 10% TNT in lanolin showed liver lesions and a positive Webster's test (a test introduced in 1916 by Webster which has been used to detect TNT metabolites in human urine in cases of intoxication). However, when Haythorn rubbed TNT powder on his arm for several consecutive days, he could not demonstrate a positive Webster's test and did not feel any ill effects.[53] In another study, TNT was rubbed into the palms of two human subjects and kept under rubber gloves for 8 hr.[54] Traces of the metabolite 2,6-dinitro-4-aminotoluene were found in the urine collected during and after the exposure to TNT.

Absorption through the respiratory system has also been examined by Haythorn.[53] When guinea pigs were exposed to fumes of volatilized TNT for 30 days, no lesions ascribed to TNT were observed, but the animals died from the heat used to volatilize TNT. In another series of experiments, TNT powder was introduced into the lungs of experimental animals, but no toxicity developed. This led Haythorn to conclude that the lung is unimportant as a route of intoxication from TNT. Later, however, Von Oettingen et al., demonstrated that 75% of a TNT dose administered to dogs by insufflation was absorbed from the respiratory tract.[48]

E. Retention and Excretion

Voegtlin et al. believed that TNT is retained in the body for a considerable period of time, as indicated by the progressive anemia after single doses of TNT and by the slow recovery of the animals.[47] However, when Von Oettingen and his co-workers administered TNT to dogs by insufflation for a period of 17 weeks, significant amounts of TNT or its metabolite, 2,6-dinitro-4-aminotoluene, were not found in any organ or tissue examined at the end of the study.[48] These authors concluded that TNT is not retained to any considerable extent in these organs. However, conclusions from these early studies regarding storage or excretion of TNT and its metabolites are hampered by the insensitivity of the methods used to examine TNT or the reduced metabolites, aminotoluenes.

Earlier studies suggested that the urine was the main route of excretion for TNT. In rats, 20% of a single oral dose of TNT was excreted in the urine as diazotizable aromatic amino compounds.[55] Human volunteers excreted an average of 40% of small oral doses of TNT as aromatic amino compounds in the urine. In other experiments, humans receiving TNT excreted about 3% of the ingested dose as 4-amino and 2,4-diamino products; concentration of these metabolites fell almost to zero within 24 hr after the last dose.[50] Although it was suggested that TNT was excreted in bile,[47] Haythorn could not obtain a positive Webster's test in the feces of animals given TNT by any route except orally.[53]

F. Metabolism

Since the beginning of this century, extensive work has been carried out to isolate and identify TNT metabolites in animals[56-58] and humans.[55,57] Only limited success has been achieved because of the difficulties encountered during the isolation procedures. Low recovery was encountered when urine samples were extracted with ether. Even after acidification of urine, no more than 15% of the administered doses were recovered in ether. The use of strong acid or base should be avoided since this undoubtedly causes alterations of the metabolites during the extraction process. 2,6,2',6'-Tetranitro-4,4'-azoxytoluene, which was reported as one of the TNT metabolites in rabbit and human urine,[57] was found later to be an artifact that was formed from the 4-hydroxylamine under the conditions of the isolation procedure. This azoxytoluene was shown to be absent from freshly voided urine of rabbits given TNT.[56]

TNT metabolism conceivably may involve alterations of the four functional groups, the open positions on the benzene ring, or scission of this ring. Ring cleavage rarely occurs and probably plays little, if any, role in the metabolism of TNT. However, a variety of other metabolic products could be formed. These may result from oxidation of the methyl group to alcohol, aldehyde, or acid; oxidation of the benzene nucleus to phenols; reduction of one or more of the nitro groups to hydroxylamino or amino compounds with the possibility of coupling of some of these metabolites; and conjugation of one or more of the resulting products (alcohols, acids, amines, hydroxylamines, etc.) to yield glucuronides, ethereal sulfates,

substituted hippuric acid, or glutathione conjugates. Simultaneous oxidation and reduction followed by conjugation is also a possibility. These hypothetical pathways, which are shown in Figure 1, illustrate the complexity of the metabolism of TNT. The problem of metabolite identification is complicated by the similar solubility characteristics possessed by these compounds of such closely related chemical structure.

Earlier studies have shown that the reduction products, 4-amino-2,6-dinitrotoluene and 2,6,2',6'-tetranitro-4,4'-azoxytoluene, are excreted in the urine of workers exposed to TNT.[42,57] Reduction of a single nitro group of TNT was also shown to occur in experimental animals leading to the formation of 4-amino- and 6-amino-dinitrotoluenes.[56] Channon et al.[56] postulated that the first step in the reduction of the nitro group is the production of a hydroxylamine derivative. The 4-hydroxylamino-2,6-dinitrotoluene was isolated as an aldoxime after reaction with benzaldehyde; the isomer 2-hydroxylamino-4,6-dinitrotoluene was not isolated, but the isolation of the reduction product 2-amino-4,6-dinitrotoluene led to the conclusion that the 2-hydroxylamine is a step in its formation.

The isolation of hydroxylamine is of interest since Wyon found the hydroxylamine derivatives to be more toxic than the parent TNT.[59] The hydroxylamine is a powerful methemoglobin producer in vitro, while TNT itself is only a weak producer of methemoglobin.[59] In addition, the formation of a hydroxylamine is implicated in the carcinogenicity responses induced by several carcinogenic amino and nitro compounds.[60] Only 1% of the TNT dose was accounted for as hydroxylamine.[56] This, however, seems to be less than the actual amount present because of the extreme ease of conversion to the azoxy derivative.

Oxidation of the methyl group of TNT may result in the formation of alcohol or acid. These oxidation processes are hypothetical and are based on some indirect evidence obtained from the studies of Channon et al.[56] Rabbits excreted some TNT metabolites as glucuronides, which were believed to arise from oxidation products of TNT such as trinitrobenzyl alcohol. However, the possibility of glucuronide conjugation with the amino or hydroxylamino derivatives was not considered. The suggestion that nitrophenylenediamine is excreted in rat urine also indicates that this oxidative pathway may be operative.[55] The loss of the methyl group could probably occur by oxidation of TNT to the alcohol, then the acid, followed by decarboxylation and reduction of the nitro group.[61] Aminonitrocresol is another oxidation product whose presence in rat urine was suggested. The mechanism of its formation is not known.

Early studies have suggested that urine from TNT workers contained the same metabolites reported in rabbit urine, namely 4-hydroxylamino-2,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, and 2-amino-4,6-dinitrotoluene.[56] Rat urine contained, in addition to the monoamines, 2,4-diamino-6-nitrotoluene and probably 5-nitrophenylenediamine.[55] On the other hand, Snyder[58] was unable to demonstrate the presence of TNT, its oxidation products (alcohol, aldehyde, or acid), or its reduction products (diamino- and triaminotoluenes) in the urine of dogs which received TNT orally.

Glucuronide conjugation appears to play an important role in the metabolism of TNT. Other conjugates and probably inorganic salts may also be formed. Channon et al.[56] found that, even after acidification of rabbit urine, no more than 15% of the administered dose was excreted as compounds soluble in ether. The ether extracts contained metabolites excreted in an unconjugated form and possibly small amounts of acetylated amino derivatives. The remainder of the doses administered were probably eliminated as conjugates, e.g., glucuronides and sulfates. The excretion of compounds in combination with glucuronic acid was suggested based on an increase in glucuronides in urine after TNT dosing.

In vitro experiments suggested that the liver is a major site for TNT biotransformation.[62] Studies using liver, muscle, and heart preparations showed that TNT was reduced by liver homogenates to 4-amino-2,6-dinitrotoluene. The rate of reduction was more rapid under anaerobic conditions. TNT metabolism occurred in a system containing reduced nicotinamide dinucleotide (NADH) and a purified flavoprotein. It was also suggested that TNT was reduced to hydroxylamines by xanthine oxidase.

III. MATERIALS

A. Animals

The adult male and female animals used in these studies were obtained from commercial suppliers. Swiss albino CD_1® mice (20 to 30 g) and Sprague-Dawley CD® rats (200 to 300 g) were purchased from Charles River Breeding Laboratories (North Wilmington, Massachusetts). New Zealand rabbits (3 to 4 kg) were purchased from Small Stock Industries (Pea Ridge, Arkansas). Beagle dogs (8 to 14 kg) were purchased from Hazleton Research Animals (Cumberland, Virginia). All animals were kept for at least 1 week prior to dosing in temperature-controlled (74 ± 2°F) and humidity-controlled (40 to 60%) rooms maintained on a 12-hr light and dark cycle. Diet consisted of commercially available rodent, rabbit, and dog chow.

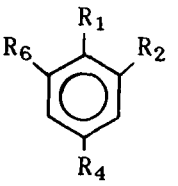
B. Chemicals

1. Test compound: Military grade 2,4,6-trinitrotoluene (TNT) was supplied by Mr. Ralph Hauze of the Volunteer Army Ammunition Plant (Chattanooga, Tennessee). Gas-liquid chromatography (GLC) analyses indicated that the test compound contained 99.82% TNT and 0.18% 2,4-dinitrotoluene (DNT).

Radiolabeled TNT-(ring-UL-^{14}C) with specific activity of 19.76 μCi/mg was purchased from Pathfinder Laboratories (St. Louis, Missouri). The radiochemical purity was found to be greater than 98% as determined by thin-layer chromatography (TLC).

2. Reference standards: Reference standards of some potential TNT metabolites were purchased from K&K Laboratories (Plainview, New York), synthesized in MRI laboratories, or obtained from Dr. N. E. Burlinson of Naval Surface Weapons Center (NSWC, White Oak, Silver Spring, Maryland). These standards are shown below.

The dinitrohydroxylaminotoluenes were prepared by reduction of TNT with a mixture of ammonium hydroxide and hydrogen sulfide according to the method of Elvove.[63] Examination of the product by TLC revealed a mixture of two major products. Attempts to separate these products by gravity column chromatography, using silica gel with various solvents, were unsuccessful. The mixture was resolved by the use of high performance liquid chromatography (HPLC) on a Woelm silica gel column. Elution with 25% petroleum ether in methylene chloride gave a yellow solid, m.p. 169-170°C. TLC on silica gel G using chloroform as the developing solvent gave a single spot with an R_f of 0.7. Further elution with methylene chloride gave a second solid, m.p. 148-149°C (R_f = 0.5 in the same system). Spectral analysis of the two products by nuclear magnetic resonance confirmed that both were dinitrohydroxylaminotoluenes, but the location of the hydroxylamine group in each was uncertain. TLC comparison with authentic samples of the two hydroxylamines obtained from Dr. N. E. Burlinson of NSWC indicated that the product with m.p. 148-149°C was 2,6-dinitro-4-hydroxylaminotoluene and the compound with m.p. 169-170°C was 2,4-dinitro-6-hydroxylaminotoluene.



	Standard	R_1	R_2	R_4	R_6
1.	Trinitrotoluene (TNT)	CH_3	NO_2	NO_2	NO_2
2.	Trinitrobenzyl alcohol	CH_2OH	NO_2	NO_2	NO_2
3.	Trinitrobenzoic acid	COOH	NO_2	NO_2	NO_2
4.	1-Amino-2,6-dinitrotoluene	CH_3	NO_2	NH_2	NO_2
5.	2-Amino-4,6-dinitrotoluene	CH_3	NH_2	NO_2	NO_2
6.	4,6-Diamino-2-nitrotoluene	CH_3	NO_2	NH_2	NH_2
7.	2,6-Diamino-4-nitrotoluene	CH_3	NH_2	NO_2	NH_2
8.	4-Hydroxylamino-2,6-dinitrotoluene	CH_3	NO_2	NHOH	NO_2
9.	2-Hydroxylamino-4,6-dinitrotoluene	CH_3	NHOH	NO_2	NO_2
10.	2,6,2',6'-Tetranitro-4,4'-azoxytoluene	CH_3	NO_2	a	NO_2

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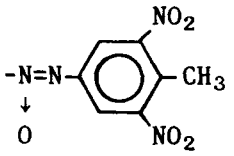
a 

Attempts to prepare 2,4,6-trinitrobenzyl alcohol by reducing the corresponding acid with boranemethyl sulfide gave a mixture of four products. Therefore, a modification of a procedure by Ganguly[64] was used. It involved treatment of TNT with sodium hypobromite to give the trinitrobenzyl bromide, followed by hydrolysis to the alcohol. Infrared and TLC analyses indicated that the alcohol was identical to a reference sample of 2,4,6-trinitrobenzyl alcohol obtained from NSWC.

Attempts to synthesize 4-amino-2,6-dinitrobenzyl alcohol by selectively reducing the 4-nitro group of the 2,4,6-trinitrobenzyl alcohol using ammonium hydroxide-hydrogen sulfide resulted in a mixture of products; attempts to purify this mixture were unsuccessful. In another experiment, the alcohol was reduced with hydrogen sulfide in dioxane.[65] This also produced a mixture of three products which proved difficult to purify.

IV. AEROSOL PRODUCTION

A. Particle Size Reduction

1. Ball milling: The first approach to reducing TNT to "respirable" size particles was to utilize conventional ball milling techniques. Approximately 200 g of military grade TNT was placed in a ball mill jar with 28 to 32 porcelain balls. The TNT was milled for varying lengths of time up to 2 hr, and samples were removed at different intervals for microscopic determination of particle size. The minimum particle size range achieved was 20 to 28 μm after 30 min of ball milling. Ball milling for longer periods of time did not result in a further reduction in particle size. After ball milling, the TNT had a tendency to clump together in the jar and adhere to the sides of the mill jar.

2. Nebulization of TNT: Nebulization of TNT from a TNT-acetone solution was attempted to produce respirable size TNT particles. This method utilized the FK-8 nebulizer gun developed at Edgewood Arsenal. The FK-8 gun is designed to produce an aerosol at the rate of 2 ml/30 sec or 240 ml/hr with a particle size of 1 to 2 μm. For the TNT experiments, nitrogen at a pressure of 60 to 70 psi was used to produce the TNT particles from a TNT-acetone solution. For these studies, the aerosol from the FK-8 gun was directed into a 5-gal. widemouthed glass jar.

Using the FK-8 nebulizer gun, it was possible to produce TNT particles in the 1- to 2-μm size range as determined from microscopic examination. However, the TNT particles produced had the tendency to adhere to the sides of the glass container. In addition, it appeared that the separation of the TNT particles from the acetone would pose a significant problem in producing an aerosol suitable for the exposure of animals.

3. Aspirator method: A TNT-acetone solution was dispersed through a cold water aspirator vortex. The TNT particles were precipitated in the water and filtered. The filtered particles were then washed with 95% ethanol followed by an ether wash to obtain dry particles. The dry TNT particles were then sized by light microscopy and were found primarily to be greater than 20 μm in size. Therefore, the aspirator method was not suitable for producing TNT particles of respirable size.

4. Ball milling in a cold atmosphere: As discussed above, conventional ball milling techniques produced TNT particles in the 20- to 28-μm size range. It was felt that the softness of TNT (a hardness of 1.4 on the Mohs scale) might have contributed substantially to the failure to produce smaller particles by ball milling. By hardening the TNT particles, it was anticipated that ball milling might result in a further reduction in TNT particles. To harden the TNT particles, ball milling in a cold atmosphere was attempted.

The initial approach to producing a cold atmosphere was to add dry ice to the ball mill jar. Although the addition of dry ice in the TNT reduced the temperature of the jar initially, the ball milling procedure

quickly dissipated the dry ice and the jar rapidly returned to ambient temperature. In addition, adding the dry ice to the jar resulted in a problem with moisture collection which in turn appeared to aggravate the clumping problem. The particle size range produced by this method was essentially the same as that obtained with conventional ball milling, 20 to 28 μm.

A second approach involved adding liquid nitrogen to the TNT. Upon addition of the liquid nitrogen to the mill jar, the TNT powder present froze into a solid sheet. After 20 min of ball milling, the particles examined microscopically were greater than 15 μm in size. As with the dry ice method, the liquid nitrogen quickly dissipated, resulting in a rapid return to ambient temperture.

To keep the ball mill jar at a reduced temperature throughout ball milling, the jar was immersed in a dry ice-acetone bath. Using this approach, it was possible to maintain the ball mill jar at a reduced temperature throughout the milling procedure. After ball milling for 1 hr in the dry ice-acetone bath, the particles obtained were greater than 10 μm in size. Ball milling in the dry ice-acetone bath for additional lengths of time did not further reduce the TNT particle size.

5. Jet pulverizer system: The jet pulverizer system (Jet Pulverizer Company, Palmyra, New Jersey) was attempted to reduce TNT to the 1-μm particle size. The jet pulverizer is a fluid energy mill in which the fluid energy (in this case nitrogen gas) is admitted in fine, high velocity streams around the periphery of a grinding and classifying chamber. The high order of turbulence created causes the particles to grind upon themselves and be ruptured, forming smaller particles.

After determining that substances of the softness of TNT could be ground successfully with the jet pulverizer system, experiments were undertaken to reduce the TNT to the desired particle size. The TNT was fed to the pulverizer using a vibrating trough which in turn was fed by a vibrating feed hopper in order to maintain a constant feed of material to the pulverizer.

It was found that TNT particles 1 to 3 μm in size could be produced using the jet pulverizer. Microscopic examination of these particles showed the particles to be spherical and 1 to 3 μm in diameter. The particles existed as both single particles and agglomerates of the 1- to 3-μm particles. After setting for a number of days, the TNT particles again displayed the tendency to clump together, although the individual particles remained 1 to 3 μm in size. Therefore, it appears the jet pulverizer is suitable for reducing TNT to a 1- to 3-μm particle size.

B. Aerosol Generation

1. Generation from jet pulverizer-produced particles: After obtaining respirable (1 to 3 μm) TNT particles with the jet pulverizer, attempts were made to produce a suitable aerosol for animal studies. Pilot

studies of aerosol generation centered on two methods. The first method involved the nebulizing of TNT suspensions. The TNT was suspended in either Tween-80 or propylene glycol. For these pilot studies, a 5-gal. widemouthed glass jar was used as the chamber in which to produce the aerosol. While the nebulization of TNT from the above suspensions will produce an aerosol cloud of TNT particles in the 1- to 3-μm range, several problems are evident. It appears that the concentrations needed to conduct the animal inhalation studies are so high that the TNT particles coalesce into larger particles and thus will not remain in airborne suspensions. Also, the TNT particles have a tendency to adhere to the surfaces of the glass jar. The net result of these problems is to greatly reduce the inhalable concentration of TNT within the chamber.

The second method of generation involved the dispersion of TNT using an air jet. As with the nebulization method, the problems of coalescence and adherence to the glass surfaces occurred. It would appear that the physicochemical properties of TNT may be at least a part of the problem because using the same method as described above, it is possible to produce an aerosol of talc which will remain in airborne suspension and will not adhere to surfaces in the manner observed with TNT.

2. Aerosolization by heating of TNT: TNT powder (20 to 28 μm in size) contained in a glass petri dish was placed on an aluminum heating plate warmed by a 175-W heater. The temperature of the heater was controlled by a thermocouple connected to a temperature controller. A second thermocouple connected to a Pyrotest meter was placed in the petri dish to allow for direct temperature readings of the melted TNT liquid.

The experiments were carried out in a 0.5 m^3 stainless steel exposure chamber. The heating device was placed in the bottom of the chamber, and the airflow entered the chamber at a point below the heating device. The airflow (50 liters/min) was controlled by a rotameter-type flowmeter. Samples for analysis of TNT concentration in the chamber were drawn through a Millipore filter (0.8-μm pores) by a vacuum pump connected to a flowmeter to control the volume of the air sample withdrawn from the chamber. The TNT was eluted from the filters with toluene and analyzed by gas chromatography. Samples were collected with an impaction device and examined by light microscopy to determine particle size.

Initial experiments confirmed that a TNT aerosol could be produced by heating the TNT to approximately 200°C. However, the actual chamber concentrations measured were only 10 to 20% of the calculated concentration, which was based on chamber airflow and the amount of TNT consumed during the experiments. The particle size of the TNT obtained by this method was principally in the 3- to 8-μm size range.

In an effort to determine the reason for the discrepancy between actual and calculated chamber concentrations, several experiments were conducted. To ensure that the chamber airflow was correct, the flowmeter was recalibrated using an Autotronics 100-SSX airflow transducer. This showed the flowmeter calibration to be correct; therefore, the airflow was not the

source of discrepancy in concentrations. Other possibilities considered included the possible generation of TNT vapor or TNT particles of smaller size that would not be captured on the Millipore filter sampling system. To test these possibilities, the chamber sampling was performed by passing chamber air samples through a vessel containing toluene to capture any TNT vapor or very small TNT particles. The concentrations obtained by this method were the same as those using the Millipore filter system. This suggests that TNT vapor or small TNT particles could not account for the discrepancy in concentrations.

As with the experiments using particles obtained with the jet pulverizer, there was a problem with TNT adhering to the walls of the exposure chamber. Also, after each experiment with the heating method, deposits of TNT dust were found in the bottom cone of the exposure chamber. In retrospect, it appears that the TNT produced by the heating method was of sufficiently high concentration to result in coalescence of particles to form larger particles which settled to the bottom of the chamber, resulting in reduced concentrations of TNT in the chamber environment. The presence of TNT particles primarily in the 3- to 8-μm range might be a further indication of coalescence. Earlier small-scale studies of the heating method had produced particles in the 1-μm size range.

C. Discussion

It appears that methods are available which can produce 1- to 3-μm TNT particles and generate TNT aerosol. However, the problem in the present study appears to be the necessity for producing extremely high concentrations of TNT which are suitable for metabolic studies. We have estimated that TNT concentrations of 1 to 2 g/m^3 would be needed to produce levels of TNT in the experimental animals that could be detected by available analytical methods. Production of TNT concentrations of this high magnitude was not possible using the methods described herein.

An alternative approach was the study of TNT disposition and metabolism after direct instillation into the trachea. This method has been used successfully by different investigators to study the toxicity and metabolism of various environmental chemicals, especially polycyclic aromatic hydrocarbons.[66-68] While this method is not an inhalation exposure in the strictest sense, it does allow for the absorption of TNT via the lung. In this way, the metabolism of the TNT can be studied under conditions in which the TNT passes through the lung prior to entry into the bloodstream. Therefore, it would appear that a valid comparison or at least approximation of TNT metabolism by oral and pulmonary absorption could be made.

V. DISPOSITION STUDIES

A. Methods

1. Oral administration: Four rats, seven or eight mice, and three rabbits and dogs of both sexes were used in these studies. The animals were fasted overnight before receiving single oral doses of ^{14}C-TNT. Rats and mice received ^{14}C-TNT doses of 100 mg/kg dissolved in 10 ml/kg (rats) or 25 ml/kg (mice) of oil. Rabbits and dogs received ^{14}C-TNT doses of 5 mg/kg body weight dissolved in 1 ml/kg oil. The dosing solutions were prepared by dissolving the appropriate amounts of nonlabeled and ^{14}C-labeled TNT in peanut oil. A fresh dosing solution was prepared for each experiment to avoid possible decomposition. When not in use, the dose was stored refrigerated at 4°C. Aliquots of each solution were counted to determine the amounts of radioactivity. All dosing solutions contained ≅ 25 μCi of the labeled compound per kilogram body weight.

After dosing, the animals were placed in individual stainless steel metabolism cages for the separate collection of urine and feces and were given food and water ad libitum. After 24 hr, the animals were anesthetized with ether (rats and mice) or sodium pentobarbital (40 mg/kg, i.p. for rabbits; and 30 mg/kg, i.v. for dogs). Blood was collected, and the following tissues and organs were removed, weighed, and analyzed for radioactivity:

Liver	Brain
Kidneys	Skeletal muscle
Lungs	Fat (retroperitoneal)
Spleen	GI tract plus contents

2. Dermal application: The fur on the back of the test animals was removed with an electric clipper. The day following clipping, the ^{14}C-TNT solution was spread over the depilated areas (2 to 4 cm^2 in mice, 8 to 10 cm^2 in rats, 150 to 200 cm^2 in rabbits, and 200 to 300 cm^2 in dogs). The doses applied were 50 mg/kg of TNT containing ≅ 25 μCi/kg of ^{14}C-TNT in 2 ml/kg of peanut oil for rats; and in 5 ml/kg for mice. Rabbits and dogs were treated with either 5 or 50 mg/kg of TNT containing ≅ 25 μCi/kg of ^{14}C-TNT in 0.5 ml/kg of peanut oil. Concurrent experiments were performed in animals treated orally with the same dose of ^{14}C-TNT and housed under the same conditions. Before being placed in metabolism cages, a plastic collar was placed around the neck of each mouse, rabbit, and dog to prevent them from grooming their fur. After dosing, rats were placed in individual restrainers, mice in small glass metabolic cages, and rabbits and dogs in steel metabolic cages. Urine and feces were collected separately for 24 hr. Blood samples were obtained from the tail veins of rats at 4, 8, and 24 hr after dosing. After 24 hr, the animals were anesthetized with ether (rats and mice) or with sodium pentobarbital (rabbits and dogs). Blood samples were collected, and the animals were killed for tissue sampling as described for the oral studies. Skin including the site of application was not retained for analysis.

The dermal and oral studies were performed with three (oral) and six (dermal) rats of both sexes, eight (oral) and seven (dermal) male mice, three (oral) and four (dermal) male rabbits, three (oral) and three (dermal) male dogs. In addition, limited studies were performed using four male rabbits (two oral and two dermal) and two male dogs (one oral and one dermal) which were treated with higher (50 mg/kg) doses of ^{14}C-TNT.

3. Intratracheal instillation: Rats were anesthetized with sodium pentobarbital (45 mg/kg, i.p.), then tracheotomized with polyethylene tubing (PE-210). The femoral artery was cannulated with PE-50 tubing for collection of blood samples.[69] After the rats were allowed a 10- to 15-min recovery period, 50 mg/kg of TNT containing ≅ 25 μCi/kg of ^{14}C-TNT was administered either orally or intratracheally. The TNT with particle size of 1 to 3 μm was suspended in a volume of 0.5 ml/kg of methylcellulose. Blood samples (0.2 to 0.3 ml) were collected for subsequent analysis. At the end of 4 hr, the rats were sacrificed, and tissues and bladder urine were collected for radioactivity analysis (see oral administration).

Since a major portion of the intratracheally administered TNT dose was recovered in the GI tract, some experiments were performed in rats which had the common bile duct cannulated with PE-10 tubing.[69] Bile was collected at different time periods after dosing and sampled for analysis. Blood samples were also collected, and the rats were sacrificed at 4 hr for tissue sampling.

4. Sample preparation and analyses: Feces and GI tract plus contents were weighed and homogenized separately in 10 volumes of ethanol:water (20:80) in a Waring blender. Duplicate aliquots of the homogenates (500 μl), whole blood (100 μl), and tissues (50 to 120 mg) were used for analysis. These samples were processed by heating in a shaking water bath at 70°C for 30 min with 0.2 ml of 70% perchloric acid and 0.4 ml of 30% hydrogen peroxide, then cooled and mixed with scintillation cocktail. (Preliminary studies indicated good recoveries when this technique was used for processing tissues and excreta.)[70,71] Volumes of urine and cage rinse were measured, and aliquots (100 μl) were mixed with scintillation cocktail. Samples were analyzed in duplicate whenever possible. Phase Counting Solution (PCS, Amersham Corporation, Arlington Heights, Illinois) was used as the scintillation cocktail.

5. Radioactivity measurements: The samples were cooled for a minimum of 24 hr before counting in a liquid scintillation counter (Packard Tricarb Model 3375). Correction for background was carried out automatically on the counter. Background determinations were obtained by averaging the natural counts of several tissue homogenates from nontreated animals. The counting efficiency was determined using the automatic external standard (AES) method. An AES versus efficiency curve was prepared by processing a quench curve set through the counter under the conditions used throughout the experiment. Assays not within ± 10% of the mean of the duplicates were reassayed in duplicate except when the sample was not available or when radioactivity counts were low and nonsignificant, i.e., less than two times the background.

6. Data processing and analysis: Carbon-14 contents in blood and tissues were presented in terms of microgram equivalents per milliliter (blood and bile) or gram (tissues) and percentage of the dose administered to each animal. Microgram equivalents per milliliter of blood and bile were also presented in graphic form. The means ± standard errors were calculated for each test group with a programmable (Monroe) calculator. The significance of the data was determined by the two-tailed Student's t test. Significant differences were indicated when $p < 0.05$.

B. Results

1. Oral studies: The disposition of orally administered ^{14}C-TNT was studied in male and female rats, mice, rabbits, and dogs. No attempt was made to examine the radioactivity in the expired air since earlier studies performed at MRI* have demonstrated that only a negligible amount (0.1%) of the administered ring-^{14}C-labeled TNT dose was eliminated by this route.

a. Rats: Recovery of radioactivity in tissues and excreta of rats is summarized in Table 1. At the end of 24 hr, a total of 52.7% of the administered dose was recovered in the urine, 8.1% in the feces, and 29.8% remained in the GI tract of male rats. Blood, liver, kidney, and spleen demonstrated high concentrations of radioactivity. The distribution and excretion of TNT and its metabolites in female rats were similar to that in male rats. During the same period the female rats excreted 64.6% of the dose in the urine and 2.1% in the feces while 33.9% remained in the GI tract. Urine of both males and females was bright red.

b. Mice: Table 2 summarizes the tissue distribution and excretion of radioactivity in mice treated orally with ^{14}C-TNT. In 24 hr, male mice excreted 41.9% of the administered dose in the urine and 22.0% in the feces; 13.5% remained in the GI tract. The females eliminated 42.9% in the urine and 9.0% in the feces; 7.4% was recovered in the GI tract. Tissues of female mice demonstrated lower radioactivity than those of males. This difference, which was statistically significant only in blood, liver, and kidney, is probably due to the low recovery in the females. Urine of mice had a bright red color similar to that of rats.

c. Rabbits: The rabbits excreted most of the administered radioactivity in the urine (66.3% of the dose in males and 78.9% in females).

* Lee, C. C., J. V. Dilley, J. R. Hodgson, D. O. Helton, W. J. Wiegand, D. N. Roberts, B. S. Andersen, L. M. Halfpap, L. D. Kurtz, and N. West. Mammalian toxicity of munition compounds: Phase 1. Acute oral toxicity, primary skin and eye irritation, dermal sensitization, and disposition and metabolism. United States Army Medical Research and Development Command, Midwest Research Institute Report No. 1, NTIS No. AD-B011, 150 (1975).

Feces contained 1.8% in both males and females. Recoveries in the GI tract averaged 7.5% in males and 4.7% in females (Table 3). Most tissues contained only small amounts of radioactivity. Liver, kidneys, and especially lungs had higher ^{14}C levels than did blood; lungs contained 9 times (males) or 14 times (females) the levels in blood. Rabbit urine did not contain the red pigment which was characteristic of the urine of rats and mice.

d. Dogs: Table 4 summarizes the tissue distribution and excretion of radioactivity in dogs after oral administration of ^{14}C-TNT. In males, 55.9% of the dose was excreted in the urine, 5.4% was recovered in the feces, and 10.0% in the GI tract. Females eliminated 60.2% of the dose in the urine and 16.8% in the feces while 4.4% remained in the GI tract. Expressed as percentages of the administered doses, dogs contained higher residual radioactivity than did rats, rabbits, or mice. Similar to rabbit urine, dog urine did not contain a red pigment.

A comparison of the tissue-to-blood concentration ratios in rats, mice, rabbits, and dogs at 24 hr after oral dosing with ^{14}C-TNT is shown in Table 5. High tissue-to-blood ratios were noted in liver (four species) and occasionally in kidneys and lungs (mice and rabbits). Rabbit lungs contained 9 times (males) or 14 times (females) higher ^{14}C levels than did blood. Low ratios (less than 1) were generally noted in brain and muscle and occasionally in lungs (rats).

2. Dermal studies: The absorption, tissue distribution, and elimination of ^{14}C-TNT was studied in male and female rats, male mice, male rabbits, and male dogs after dermal application. Concurrent experiments were performed in animals treated orally with the same dose of ^{14}C-TNT and housed under the same conditions used for the dermal application experiments. No attempt was made to measure the radioactivity on the site of dermal application.

a. Rats: Both male and female rats absorbed TNT after dermal application. Radioactivity in the blood increased with time following dermal application and continued to increase until at least 24 hr after dosing. After oral administration, on the other hand, the highest radioactivity in the blood was seen at 8 hr (Table 6). After both treatments, the urine of rats was red.

At the end of 24 hr, the distribution of radioactivity in blood, lung, spleen, brain, and muscle was comparable after both oral and dermal administration of ^{14}C-TNT to male rats (Table 7). However, the fat contained a higher concentration of radioactive TNT after dermal application, and the liver and kidney contained higher concentrations of radioactivity after oral dosing. Most of the absorbed radioactivity was excreted in the urine, averaging 17.4% of the administered dose after dermal application and 59.5% after oral administration. Radioactivity was also recovered in the feces and GI tract, averaging 1.3 and 3.1%, respectively, after dermal application; and 10.7 and 20.2%, respectively, after oral treatment.

In female rats, the distribution of radioactivity was similar to that in male rats (Table 8). At the end of 24 hr, the distribution of

radioactivity in blood and most tissues was comparable after oral and dermal administration. Fat contained greater levels of radioactivity after dermal application, and liver contained more radioactivity after oral dosing. These differences, however, were not significant. At the end of 24 hr, a total of 14.6, 2.5, and 6.4% of the dermally applied radioactivity was recovered in the urine, feces, and GI tract, respectively. After oral administration, recoveries from urine and GI tract were significantly greater, averaging 42.5 and 35.3%, respectively, of the administered dose; in the feces, recovery was 2.1% after oral administration.

b. Mice: After dermal application of ^{14}C-TNT to male mice, absorption occurred readily. At the end of 24 hr, 22.7, 14.2, and 3.6% of the administered dose was recovered in the urine, feces, and the GI tract, respectively (Table 9). After oral dosing, the recovered radioactivity averaged 59.1, 24.1, and 10.2%, respectively; these recoveries were significantly larger than those after dermal application. Radioactivity remaining in most tissues was comparable after both routes of administration. As in rats, ^{14}C content in fat was higher after dermal application, whereas the radioactivity in liver was higher after oral dosing. After both routes of administration, the urine had the same red color that was observed in urine of TNT-treated rats.

c. Rabbits: A dose of 5 mg/kg of ^{14}C-TNT was administered to groups of male rabbits dermally or orally. This dose was the same as was used in the oral studies performed earlier. However, the volume of vehicle (peanut oil) was reduced to 0.5 ml/kg. After dermal application, the major portion of the absorbed radioactivity was eliminated in the urine, averaging 52.9% of the dose (Table 10). In addition, 7.8% of the dose was recovered in the feces and 5.8% in the GI tract. After oral dosing, recoveries in the urine, feces, and GI tract averaged 68.1, 5.4, and 19.7%, respectively. Radioactivity in blood and residual bile was higher after oral administration, whereas radioactivity in kidney, lung, brain, and fat was higher after dermal application.

An additional study was conducted in groups of male rabbits treated dermally or orally with a 50 mg/kg dose of ^{14}C-TNT. This study was performed in order to (a) acquire larger amounts of TNT metabolites in the urine for TLC analysis; (b) compare the profiles of metabolites in different species after administration of the same dose of ^{14}C-TNT; and (c) examine the effect of increasing dose on the disposition and metabolism of TNT. Apparently the high dose, 50 mg/kg, did not alter the absorption, distribution, and excretion of TNT when compared with the low dose, 5 mg/kg (Table 11). However, the number of rabbits used (two per treatment) was too small to make a statistical comparison between the different dose levels or treatments. The red pigment excreted in the urine of rats and mice treated with a 50 mg/kg dose of TNT was not found in the urine of rabbits treated with the same dose, although it was reported earlier[56] that a red pigment was excreted in the urine of rabbits treated with higher and repetitive doses of TNT.

d. Dogs: A dose of 5 mg/kg of ^{14}C-TNT was administered to male dogs orally or applied dermally. The absorption of TNT after dermal

application was significantly lower than in rabbits and mice and slightly lower than in rats. At the end of 24 hr, 11.7% of the dose was recovered in the urine, 1.7% in the feces, and 1.7% in the GI tract (Table 12). After oral administration, 70.5% of the dose was excreted in the urine and 9.0% in the feces while 14.6% remained in the GI tract. Radioactivity in blood, liver, kidney, spleen, muscle, and residual bile was higher after oral administration, whereas radioactivity in fat was higher after dermal application.

To obtain preliminary information on the effect of dose on TNT absorption and elimination in dogs, ^{14}C-TNT was administered orally and dermally at a dose of 50 mg/kg. One animal was dosed by each route. Absorption and excretion of TNT appeared similar in both dose levels studied (5 and 50 mg/kg), although blood content (percent of dose) was higher after the high dose of TNT (Table 13).

After administration of ^{14}C-TNT to dogs by both routes, radioactivity was concentrated in the residual bile and liver (Table 14). Radioactivity levels were also high in the residual bile and liver of rabbits; levels in bile were considerably higher than in the blood. The concentration ratios (bile/liver and bile/blood) of radioactivity were higher for dogs than for rabbits (Table 14).

The tissue-to-blood concentration ratios at 24 hr after oral or dermal dosing of ^{14}C-TNT are shown in Table 15. Liver, kidney, lung, and occasionally spleen showed ratios higher than 1.0, while brain and muscles demonstrated ratios lower than 1.0. The ratios in fat differed after both routes of administration and were lower than 1.0 after oral administration and higher than 1.0 after dermal treatment.

3. Intratracheal studies: As an alternative approach to the exposure of rats to TNT by inhalation, studies were performed in which ^{14}C-TNT was instilled directly into the trachea of rats. A suspension of ^{14}C-TNT was delivered through a cannula placed surgically into the trachea in order to ensure that the precise dose was administered. Attempts to let the anesthetized rats recover failed. Therefore, subsequent experiments were performed under pentobarbital anesthesia. Although it was appropriate to administer the 100 mg/kg dose of ^{14}C-TNT used in the oral experiments described earlier, limitations on the quantities of powder and vehicle instilled into the trachea necessitated reducing the dose to 50 mg/kg and the volume of the vehicle to 0.5 ml/kg. Initially, 0.2% solution of Tween 80 was used to suspend ^{14}C-TNT, but it was found that the use of a solution of 0.5% methylcellulose was satisfactory. Concurrent experiments were performed in rats treated orally with the same dose of ^{14}C-TNT under the same experimental conditions.

Preliminary experiments performed in rats dosed intratracheally indicated a fast rate of absorption of TNT from the trachea and disappearance of TNT from blood. Therefore, the experiments were terminated 4 hr after dosing. Since the GI tracts of the intratracheally treated rats contained considerable amounts of radioactivity, some experiments were performed in which the bile ducts were cannulated for collection of bile. The survival

rate during the intratracheal instillation of TNT was fair; more than 80% of the treated rats survived the experiment. Some of the surviving rats had slight difficulty in breathing for about 10 min after dosing.

After oral administration of ^{14}C-TNT, radioactivity appeared in the blood of male rats within 15 min (Figure 2). The radioactivity in blood continued to increase for 60 min and maintained a constant level thereafter during the 4-hr experiment. After intratracheal instillation, absorption was faster, greater, and more uniform with less individual variation than was noted after oral administration. Orally treated male rats excreted 10.7% of the administered dose in the urine and 11.6% in the bile from bile duct-cannulated rats (Table 16). The amounts excreted in the urine and bile were higher after intratracheal instillation, averaging 17.5 and 19.8% of the dose, respectively. As shown in Figure 3, biliary excretion reached a peak 30 min after oral administration and remained constant thereafter. After intratracheal instillation, biliary excretion quickly reached a peak at 30 min and decreased gradually thereafter. Cumulative excretion of radioactivity in bile is shown in Figure 4. Urinary and biliary excretions were generally lower in female rats. Excretion of radioactivity in urine and bile averaged 8.4 and 9.7% of the administered dose, respectively, after oral administration; and 12.7 and 14.5%, respectively, after intratracheal instillation (Table 17). In rats without biliary cannula, excretion in the urine was higher (Tables 16 and 17). In all cases, urine was red and bile was dark orange.

High concentrations of radioactivity were found in tissues, especially in the blood, liver, kidney, lung, fat, and GI tract (Tables 16 and 17). In general, radioactivity in most tissues was higher after intratracheal instillation than after oral administration. Levels of ^{14}C in blood and tissues of female rats were about two times higher than in the males. Radioactivity was concentrated in the bile; bile-to-liver and bile-to-blood concentration ratios were high after both routes of administration (Table 18).

A comparison of the tissue-to-blood concentration ratios at 4 hr after oral or intratracheal administration of ^{14}C-TNT is shown in Table 19. Fat-to-blood ratios were high (3.2-5.3) in males and females after both routes of administration. The lungs of male rats also showed high ratios after oral and intratracheal dosing. The ratios in liver were about 1.0 in males and less than 1.0 in females. Ratios less than 1.0 were demonstrated in spleen, brain, and muscle of all treatment groups.

C. Discussion

There are three possible routes for TNT to enter the body: ingestion, absorption through the skin or via the lung, or any combination of these, depending on the type of exposure involved. Earlier studies have suggested that the skin is the chief avenue of absorption.[46] Voegtlin et al.[47] have demonstrated that in humans, skin absorption takes place readily through the hands, neck, and face; oily skin and sweat favor absorption.

Although some experiments have demonstrated that TNT is absorbed when introduced as dust in the lower air passages, Puntam and Herman[46] suggested that intoxication via the respiratory tract rarely, if ever, occurs.

1. Oral absorption: During exposure to TNT, the powder may be ingested by mouth and gain access to the stomach. TNT workers have complained about a bitter taste in their mouth. When two human subjects received daily doses of 1 mg/kg of TNT for four successive days, 3% of the total amount of TNT administered was recovered from the urine in the form of 2,6-dinitro-4-aminotoluene.[50] Experimentally, guinea pigs fed oral doses of TNT with milk developed diarrhea, and poisoning symptoms were apparent for 3 to 14 days.[51]

The present study demonstrates that TNT is readily absorbed after oral administration to rats, mice, rabbits, and dogs. It appears that the rabbits and dogs absorb more TNT than do rats and mice. However, the extent of absorption can be only approximated from our recovery data since the extent of biliary excretion and enterohepatic circulation was not studied. Radioactivity recovered in the GI tract represents a balance between absorption, biliary excretion, and intestinal reabsorption.

2. Dermal absorption: TNT was reported to be absorbed through the intact skin of swine as indicated by the presence of 2,6-dinitro-4-aminotoluene in urine.[52] Also, Haythorn reported that guinea pigs and rabbits rubbed repeatedly with 10% TNT in lanolin showed a positive Webster's test (a test introduced in 1916 by Webster which has been used to detect TNT in human urine in cases of intoxication) and liver lesions.[53] However, when Haythorn rubbed TNT powder on his arm for several consecutive days, he could not demonstrate a positive Webster's test and did not feel any ill effects.[53] In another study,[54] powdered TNT was rubbed into the palms of two human subjects and kept under rubber gloves for 8 hr; traces of the metabolite 2,6-dinitro-4-aminotoluene were found in the urine collected during the exposure and for 15 hr thereafter.

The dermal experiments performed in the present study confirm the potential absorption of TNT through the skin. TNT was most readily absorbed by rabbits, followed by mice, rats, and finally dogs. The majority of the administered dose was recovered in urine, feces, and GI tracts. In all species, the total elimination of the administered radioactivity was lower following dermal application than after oral treatment.

3. Pulmonary absorption: Absorption through the respiratory system has been previously examined by Haythorn.[53] When guinea pigs were exposed to fumes of volatilized TNT for 3 hr/day for 30 days, no lesions ascribed to TNT were observed, but the animals died from the heat used to volatilize TNT. In another series of experiments, TNT powder was introduced into the lungs of experimental animals, but no toxicity developed. This led Haythorn to conclude that the lung is unimportant as a route of intoxication from TNT. Later, however, Von Oettingen and his colleagues administered TNT to dogs by insufflation and demonstrated that 75% of the dose was absorbed from the respiratory tract.[48]

In the present study, extensive absorption was demonstrated when TNT suspension was instilled in the rat trachea. The pharmacokinetic behavior of TNT after intratracheal instillation was comparable to the behavior usually observed after intravenous administration of other xenobiotics. The rate of absorption was considerably faster than after oral administration, and blood levels also decayed at a faster rate. Intratracheal instillation of TNT was not studied in mice, rabbits, or dogs. If the results of the rat study can be extrapolated to other experimental animals and humans, it suggests that, when TNT powder reaches the respiratory tract, absorption will occur at a fast rate.

The dermal and oral studies were terminated after 24 hr, but the intratracheal instillation experiments were terminated 4 hr after dosing. Therefore, no data are available for direct comparison between the intratracheal and dermal routes. Blood sampled at 4 hr after dermal treatment of male rats showed considerably lower levels of radioactivity than the levels obtained after intratracheal dosing. However, blood levels continued to increase between 4 and 24 hr after dermal application; after intratracheal administration these levels would probably decrease. Therefore, the available data indicate that the rates of absorption and elimination of TNT are highest after intratracheal instillation and lowest after dermal application.

4. Tissue retention: Voegtlin et al. believed that TNT is retained in the body for a considerable period of time, as indicated by the progressive anemia after single doses of TNT and by the slow recovery of the animals.[47] However, when Von Oettingen and co-workers administered TNT to dogs by insufflation 5 days/week for a period of 17 weeks, significant amounts of TNT or its metabolite, 2,6-dinitro-4-aminotoluene, were not found in any organ or tissue examined at the end of the study.[48] These authors concluded that TNT is not retained to any considerable extent in these organs. Conclusions from these early studies regarding storage or excretion of TNT and its metabolites are hampered by the insensitivity of the methods used to examine the presence of either TNT or the reduced metabolites, aminotoluenes.

Although the experiments in the present studies were not extended beyond 24 hr, there is indication that retention in tissues of the four species examined is not extensive. The extent of retention and storage of radioactivity did differ, however, between species and between routes of administration. In addition, the patterns of radioactivity in tissues of rats were different when examined at 4 hr compared to 24 hr after treatment. The present studies were performed after administering single doses of TNT. It may be possible that after repetitive dosing the amounts of TNT and/or its metabolites retained in the various tissues would differ from amounts retained after single doses.

5. Urinary excretion: Based on Webster's test, it was suggested that the urine was the main route of excretion for TNT. Studies by Lamberg and Callaghan indicated that 20% of a single oral dose of TNT was excreted in the urine of rats as diazotizable aromatic amino compounds.[55] Human volunteers excreted ∿ 40% of small oral doses of TNT as aromatic amino compounds in the urine. In other experiments, humans receiving 1 mg/kg/day of

TNT excreted about 3% of the ingested dose as 4-amino and 2,4-diamino products; concentration of these metabolites fell almost to zero within 24 hr after the last dose.[50] The present studies demonstrated that after oral administration of TNT to rats, mice, rabbits, and dogs, large portions of the administered doses were excreted in the urine. After intratracheal instillation, extensive elimination occurred through the urine and the GI tract. After dermal application, dogs and rats excreted only small portions of the doses in the urine and feces within 24 hr. On the other hand, rabbits and mice excreted large portions of the doses in the urine and feces; these differences in excretion may be due solely to differences in the rates of absorption.

Urine from humans and some experimental animals given TNT contained a red pigment which was believed to be a metabolic product of TNT.[56] Rats treated with α-TNT (2,4,6-TNT) or with 2,4,6-trinitrobenzyl alcohol excreted the red pigment; β-TNT (2,3,4-TNT), γ-TNT (2,4,5-TNT), and several other related TNT intermediates did not cause any changes in color of the urine.[56] In the present studies, the bright red color of urine was observed in rats and mice treated by the different routes of administration. Urine of dogs and rabbits treated orally or dermally contained no red pigment even after treatment with high doses (50 or 100 mg/kg). This seems to be in variance with the early observations by Channon et al., who reported the presence of red pigment in the urine of rabbits treated orally with TNT.[56] Their experiments, however, were repetitive and used higher doses of TNT.

6. Biliary excretion: Voegtlin et al. suggested that TNT was excreted in bile.[47] Haythorn, however, could not obtain a positive Webster's test in the feces of any animals given TNT by any route except orally.[53] The present experiments demonstrated that in all species examined biliary excretion plays a major role in the disposition of TNT as indicated by the radioactivity recovered in the bile of bile duct-cannulated rats or in the residual bile of dogs and rabbits. It was also indicated by the excretion of radioactivity in feces and GI tract after dermal application of TNT to rats, mice, rabbits, and dogs, or after intratracheal instillation in rats.

The enterohepatic recycling of TNT and/or its metabolites (excretion in bile followed by intestinal absorption and reexcretion in urine or feces) was suggested by the higher recovery of radioactivity in the urine of noncannulated rats than in urine of bile duct-cannulated rats. In addition, radioactivity excreted in the bile of rats was equal to or more than that excreted in the urine, whereas radioactivity excreted in the urine of noncannulated rats was more than that recovered in the feces and GI tracts. Radioactivity recovered in the GI tract after oral dosing represents a balance between absorption, excretion in bile, and intestinal reabsorption. After intratracheal administration, the recovered radioactivity represents the difference between biliary excretion and intestinal reabsorption; only a small portion of the dose appears to be excreted through the intestinal wall.

VI. METABOLIC STUDIES

A. Methods

1. Extraction and cleanup procedures: For the chromatographic separation of trinitrotoluene (TNT) and its metabolites, urine samples were extracted with either ethyl acetate or ether. The efficiency of the extraction was examined at the pH of the raw urine (pH 7-8 for rat, mouse, and dog urine, and pH 9-9.5 for rabbit urine); at pH 6, 7, or 8 by adding equal volumes of the corresponding phosphate buffer (0.2 M); and at pH 4 or 5 by treatment with acetate buffers (0.2 M). Urine samples were also extracted after adding varied amounts of dilute acid (hydrochloric or acetic) or alkalis (sodium hydroxide or carbonate). In addition, some urine samples were extracted successively with ethyl acetate under acidic, neutral, and basic conditions and the extracts were pooled. Early in the study, urine samples were treated with a strong acid (5 N hydrochloric acid) and heated for 1 hr at 100°C prior to the extraction procedure. This method was abandoned later in view of the demonstrated instability of many of the metabolic products of TNT under these conditions.

It was established that acidification with 0.1 N hydrochloric acid (1/10 of urine volume) before extraction with ethyl acetate resulted in the highest recovery. Therefore, the method routinely used involved the extraction of urine or bile samples with 10 volumes of ethyl acetate after acidification with 1/10 volume of dilute hydrochloric acid (0.1 N). The samples were mixed thoroughly for 3 min with a vortex mixer and centrifuged. The organic extracts were separated, dried with anhydrous calcium chloride, and filtered. To assess the recovery of extractions, aliquots of these extracts (0.5 ml) were placed in counting vials and counted in 10 ml of scintillation cocktail. This extraction procedure was repeated three times, and the extracts were pooled and evaporated in a rotary evaporator. The residues were dissolved in small volumes (0.1 to 0.2 ml) of methanol, ethyl acetate, or a mixture of both solvents (1:1), filtered through Millipore filters, and used for analyses with thin-layer chromatograpy and high performance liquid chromatography.

Some urine samples were lyophilized (freeze-dried), and the residues were dissolved in methanol, ethyl acetate, or a mixture of both solvents (1:1). These were centrifuged, filtered through Millipore filters, and used for chromatographic analysis. Additional urine samples were purified by using XAD-2 (Amberlite) resin. The urine was passed through the resin column, and the radioactivity was eluted with water followed by methanol. Radioactivity was counted in each fraction collected.

2. Enzyme hydrolysis: During the early studies, urine samples were acidified with 5 N hydrochloric acid and heated for 1 hr to hydrolyze the conjugated metabolites. This method was abandoned later after it was recognized that the nature of TNT metabolites might be altered by this treatment. The hydrolysis of the conjugated metabolites was carried out by incubation with excess β-glucuronidase (type II, Sigma Chemical Company,

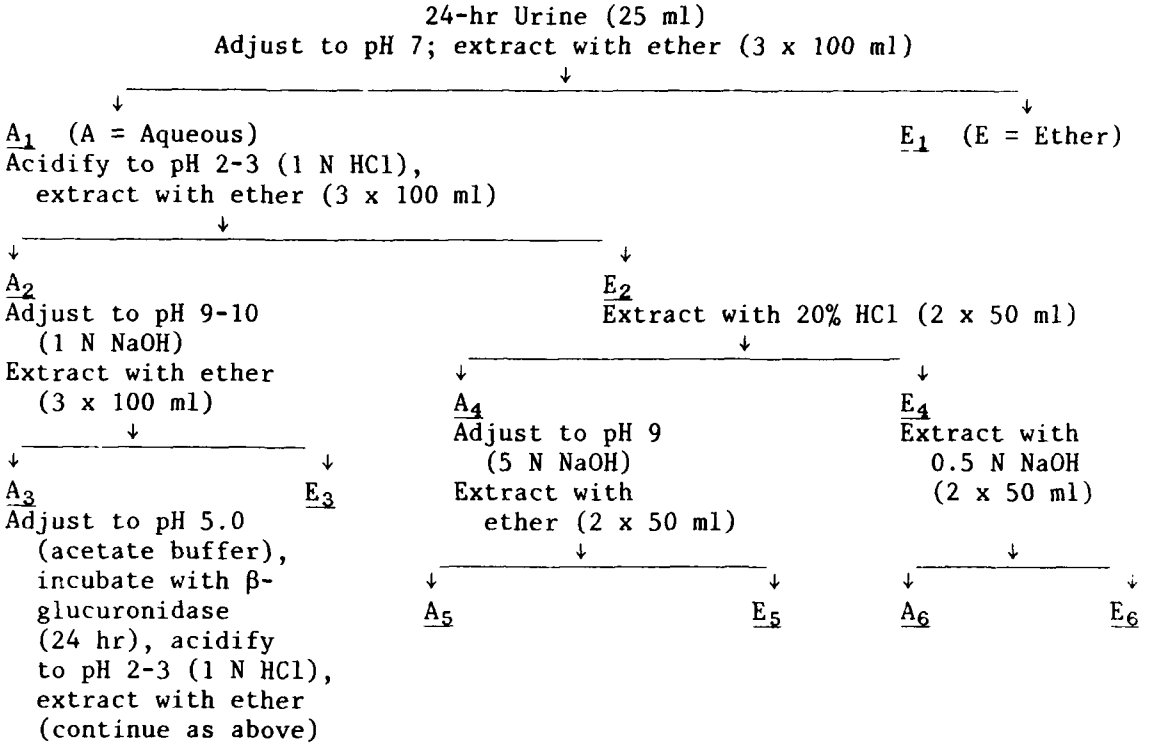
St. Louis, Missouri) at a final concentration of about 20,000 units/ml, after adjusting the urine samples to pH 5 with sodium acetate-acetic acid buffer (0.2 M). Some urine samples were also treated with aryl sulfatase (type V, Sigma) under the same pH conditions. For routine analysis, urine or bile samples (1 to 2 ml) were mixed with equal volumes of the buffer, and the mixtures were treated with 0.25 to 0.5 ml of liquid β-glucuronidase (type H-2, 100,000 units/ml) which also contains some aryl sulfatase. The reaction mixtures were incubated (37°C) under anaerobic conditions (N_2) for 24 hr in a Dubonoff shaking incubator (100 cycles/min). Urine incubated only with the acetate buffer served as control (to assess the nonenzymatic hydrolysis).

After incubation, the reactions were terminated by the addition of 0.5 ml of 0.1 N HCl and 10 ml of ethyl acetate which was used to initiate the extraction process. The aqueous layers were extracted further with two 10-ml portions of ethyl acetate. The extracts were pooled, dried with anhydrous calcium chloride, and filtered. The filtrates were evaporated under vacuum, and the residues were dissolved in 0.1 to 0.2 ml of methanol, ethyl acetate, or a mixture of both solvents (1:1). Portions of these solutions were used for the characterization of TNT metabolites with TLC or HPLC. Some urine samples were incubated with β-glucuronidase and analyzed with HPLC without prior extraction with ethyl acetate.

3. Fractionation of urinary metabolites: To simplify the chromatographic profile of TNT metabolites, separation of these metabolites into several major subgroups was attempted. Urine samples were subjected to a series of extractions with ether under different pH conditions. Several modifications were made during the development of the procedure, shown in the following diagrammatic scheme. The ether extracts were dried with anhydrous sodium sulfate, filtered, and evaporated under vacuum. The residues were prepared for TLC analysis. The remaining aqueous layer (A_3) was acidified and incubated with β-glucuronidase, and the extraction process was repeated. To calculate recoveries, portions of the ether and aqueous solutions were placed in scintillation vials, mixed with 10 ml of scintillation cocktail, and counted.

For comparison, a mixture of TNT and the nine available standards of potential metabolites listed below was subjected to the same extraction procedure. The lack of solubility of some of these metabolites (especially the azoxytoluene derivative) in water necessitated the addition of small amounts of methanol (5% of the total volume). After extraction, the different ether fractions were evaporated, and the residues were subjected to TLC analysis.

1. Trinitrotoluene (TNT)
2. Trinitrobenzyl alcohol
3. Trinitrobenzoic acid
4. 4-Amino-2,6-dinitrotoluene
5. 2-Amino-4,6-dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2',6'-Tetranitro-4,4'-azoxytoluene



4. Thin-layer chromatography: Precoated silica gel G plates (0.25 mm thickness on aluminum support) purchased from Brinkmann Instruments, Inc. (Des Plaines, Illinois) were used routinely throughout the study. Samples of urine or bile extracts and raw or lyophilized urine were spotted (≅ 2.0 cm from the bottom of the plate) and developed for 15 to 16 cm. The following solvent systems were tested for their ability to separate TNT and some reference standards of potential metabolites:

Solvent I:	n-Butanol:acetic acid:water (10:1:1, v/v/v)
Solvent II:	Benzene:acetic acid (9:1, v/v)
Solvent III:	Toluene:benzene:hexane (10:10:5, v/v/v)
Solvent IV:	Ethyl acetate:n-heptane (9:1, v/v)
Solvent V:	Benzene:ethyl acetate (4:1, v/v)
Solvent VI:	n-Butanol:acetic acid (10:1, v/v)
Solvent VII:	Benzene:acetic acid (4:1, v/v)
Solvent VIII:	Benzene:ethyl acetate (2:1, v/v)
Solvent IX:	Toluene:acetic acid (4:1, v/v)

For routine analysis, solvent systems I and VII were used for developing different TLC plates. Due to the Occupational Safety and Health Administration (OSHA) restrictions on the use of benzene, solvent system VII was replaced later with system IX, which contains toluene. Samples of pure TNT and nine reference standards of potential metabolites (the alcohol,

acid, monoamines, diamines, hydroxylamines, and azoxytoluene) were spotted and developed alongside the extracted material. After development, the plates were air-dried, cut into 1.0 cm zones (unless otherwise specified), and placed in scintillation vials. Ten milliliters of scintillation cocktail (PCS, Amersham) were added, and the vials were mixed thoroughly with a vortex mixer and counted.

Occasionally, the dried plates were sprayed with Bratton-Marshall reagent to detect the presence of arylamines.[72] Nitro compounds were detected by using 5% diphenylamine in absolute ethanol.[73] The presence of hydroxylamines was examined by spraying the plates with triphenyltetrazolium chloride (TTC) in the presence of alkali or with Benedict's reagent.[74] Hydroxylamines developed a purple red color in TTC and were mildly reducing to Benedict's reagent.

5. Gas-liquid chromatography (GLC): A Hewlett-Packard Model 5736-A gas chromatograph equipped with a flame ionization detector was used for GLC. Two columns, differing in polarity, were tested for the separation of TNT and potential metabolites. Column A was a stainless steel column (0.125 in. ID x 3 ft) packed with 10% UCW-872 on WAW-DMCS (80-100 mesh); column B was a glass column (0.25 in. ID x 4 ft) packed with 1.5% DC LSX-30295 and 1.5% GE-XE-60 on Gas Chrom Q (60-80 mesh).

6. High performance liquid chromatography: A Waters Associates liquid chromatograph equipped with a Model U6K injector, Model 660 programmer, Model 6000 pumps, and Model 440 detector (254 nm, ultraviolet) was used. The following four HPLC systems were examined for their ability to separate TNT and some of its potential metabolites. System 1 was regular phase chromatography, but systems 2 to 4 utilized the counter-ion reverse phase chromatography. System 4 was selected for the analysis of urinary metabolites.

System 1: Column: μ Porasil, 300 x 4 mm ID
Solvent: Isocratic tetrahydrofuran (THF):hexane (5:95) for 5 min, THF:hexane (5:95 to 45:55) in 15 min, after 30 min programmed from THF:hexane (45:55 to 70:30) in 5 min
Flow rate: 1 ml/min

System 2: Column: C_{18} μBondapak, 300 x 4 mm ID
Solvent: A--0.005 M tetrabutylammonium hydroxide (TBA) in water adjusted to pH 7.5 with phosphoric acid
B--0.005 M TBA in tetrahydrofuran, to which is added the same amount of phosphoric acid as used in solvent A
Flow rate: 1 ml/min
Program: 0 to 100% B in 15 min
Program type: 6 (linear)

System 3: A modification of system 2 to obtain better resolution.

Column: C_{18} μBondapak, 300 x 4 mm ID
Solvents: A--0.005 M TBA in water adjusted to pH 7.5 with phosphoric acid
B--0.005 TBA in methanol to which is added the same amount of phosphoric acid used in A
Flow rate: 1 ml/min
Program: Isocratic 25% B for 30 min, then 25 to 100% B in 25 min
Program type: 9 (nonlinear)

System 4: A modification of system 3 developed to retain the same resolution but to allow the polar urine components (excipients) to be eluted at the solvent front before elution of the compounds of interest. The following modifications were made in system 3:

Program: Isocratic 15% B for 20 min, then 15 to 100% B in 30 min
Program type: 6 (linear)

Samples of urine or urine extracts (20 to 100 μl) were injected into the system, and fractions of 0.4 to 0.8 ml were collected for liquid scintillation counting.

B. Results

1. *Extraction and cleanup procedures*: Extraction of the urine with ether or ethyl acetate without adjusting the pH resulted in very low recovery of radioactivity. The recovery was not greatly improved by treating the mixture with 0.2 M phosphate buffers (pH 6, 7, or 8), but addition of acetate buffer (pH 4.0 or 5.0) increased the amounts extracted. A considerable increase in the recovered radioactivity was achieved by the use of dilute (0.1 N) HCl (1/10 volume of urine sample) followed by extraction with ethyl acetate or ether. No additional radioactivity was obtained when a stronger acid was used; in fact, this reduced the amount of extractable radioactivity in the organic solvent. In alkaline medium (NaOH or Na_2CO_3), very little radioactivity was recovered. When urine samples were extracted successively with ethyl acetate under neutral, alkaline, and acidic conditions and the extracts combined, the recovery was only slightly higher than that obtained under mild acid conditions. The method routinely used for urine or bile extraction involved the acidification with 0.1 N HCl (1/10 volume) and extraction with ethyl acetate three times. A large portion of the radioactivity was, however, still unextractable under these conditions.

When urine samples were lyophilized and the residues dissolved in methanol, ethyl acetate, or a mixture of both solvents, it was noted that a

major portion of the radioactivity was not soluble. Attempts were also made to purify urine samples by passing them through a column of XAD-2 (Amberlite) resin and eluting with water followed by methanol. It was hoped that the radioactivity would be eluted only with methanol. This, however, was not the case; the major portion of radioactivity was eluted with water. Therefore, this procedure was abandoned.

2. Enzyme hydrolysis: Urine samples from rats, mice, rabbits, and dogs were hydrolyzed by incubation with β-glucuronidase (free of aryl sulfatase) in the presence of acetate buffer, pH 5.0. At the end of incubation, samples were acidified with 0.1 N HCl and extracted with ethyl acetate. Considerable increases in the extractable radioactivity occurred after hydrolysis. These increases were not detected when incubations were performed in the presence of both β-glucuronidase and saccaro-1,4-lactone (10 μm), an inhibitor of the β-glucuronidase enzyme. This indicated that the hydrolysis of the glucuronide conjugates was enzymatic. Incubation with aryl sulfatase did not increase the amount of extractable radioactivity. Routine hydrolysis was carried out with β-glucuronidase which contained some aryl sulfatase.

The amounts of ethyl acetate-extractable radioactivity from urine incubated with or without β-glucuronidase are shown in Table 20. The amounts of extractable radioactivity from urine of each species were not different after oral or dermal administration or after oral or intratracheal administration of TNT. Extractable radioactivity was also not different in male or female rats. Without incubation with β-glucuronidase, the urine from mice contained more extractable radioactivity than urine from rabbits, dogs, or rats. Incubation with β-glucuronidase increased the extractable radioactivity of urine samples from all species regardless of route of administration. The ratios of extractable radioactivity after incubation with β-glucuronidase to that without incubation with β-glucuronidase were low for urine from mice and high for urine from rats, rabbits, and dogs. These results suggest that the urine from mice contained small amounts of glucuronide conjugates of TNT metabolites and that the urine from rats, rabbits, and dogs contained large amounts of the conjugates.

Bile samples from rats, rabbits, and dogs were also extracted with ethyl acetate after incubation without or with β-glucuronidase. The results indicate that the amount of radioactivity extractable in ethyl acetate without hydrolysis was small in bile from the three species. Considerable increases in the extractable radioactivity occurred after incubation with β-glucuronidase, suggesting that bile contained large amounts of glucuronide conjugates. No major differences were demonstrated in the amounts extracted from bile of orally or intratracheally treated rats, and orally or dermally treated rabbits and dogs (Table 21).

3. Fractionation of urinary metabolites: Because of the closely related chemical structures, the similar solubilities, and the amphoteric nature of several metabolic products of TNT, complete separation of these metabolites would not be expected to occur by simple extraction. Therefore, a method was worked out to separate the metabolites in urine into several subgroups according to their solubility in organic and aqueous solvents at different pH conditions.

A mixture of TNT and nine potential metabolites was subjected to the extraction procedure. The organic fractions were analyzed with TLC to determine the recovery of each compound in each fraction. As shown in Figure 5, TNT and some potential metabolites were extractable to different extents in ether (E_1) before acidification. None of the trinitrobenzoic acid was extractable until after acidification. In this fraction (E_2), all the acid and most of the monoamines, hydroxylamines, alcohol, and azoxytoluene were recovered. Some of the diamines and TNT were also present. Most of the diamines, however, and some of the monoamines were present in the basic fraction, E_3. When the E_2 fraction was extracted with 20% hydrochloric acid, all the monoamines and diamines present in this fraction were removed into the acid (A_4). Ether extraction of A_4, after alkalinization with NaOH, removed all the monoamines and diamines into E_5. Compounds remaining in E_4, which are the acid, most of the hydroxylamines, alcohol, and azoxytoluene, in addition to some TNT, were extracted with NaOH. The acid and most of the hydroxylamines were removed into the aqueous fraction, A_6. The remaining ether extract (E_6) contained the alcohol, the azoxytoluene, some of the hydroxylamines and TNT.

Results from Figure 5b show that the E_1 fraction of the urine samples contained very low radioactivity, ranging from 4.6% in rat urine to 11.0% in dog urine obtained after oral administration of TNT. The extractable radioactivity was higher in urine of rats (10.7%) and dogs (16.1%) obtained after dermal application. The extractable radioactivity from urine of mice and rabbits remained about the same after both routes of administration. In all species, most of the radioactivity remained in the aqueous solution, A_1. Acidification of this solution with dilute HCl increased considerably the recovery of radioactivity in the ether extract. Recoveries from urine of orally treated animals in the E_2 fraction ranged from 32.6% for rabbits to 51.4% for mice. The amounts extracted from urine of dermally treated animals ranged from 22.8% for dogs to 38.5% for mice and rabbits. About 40 to 60% of the initial radioactivity in urine samples remained in the aqueous solution, A_2.

When this solution (A_2) was adjusted to pH 9-10 and extracted with ether, only small portions of the radioactivity were extractable (E_3). These ranged from 1.4 to 2.6% for urine obtained after oral administration and 0.6 to 1.9% for urine obtained after dermal application. The aqueous fractions, A_3, which seemed to contain conjugated metabolites, were treated with acetate buffer (pH 5.0) and incubated with β-glucuronidase, then subjected to ether extractions as described above. The extraction performed after hydrolysis with β-glucuronidase was attempted with urine samples from rats and rabbits. However, after it was apparent that the metabolic profiles after β-glucuronidase hydrolysis were almost identical to those obtained without β-glucuronidase treatment, successive extraction and analysis of metabolites after hydrolysis were discontinued.

The radioactivity in the E_2 fractions was extracted with 20% HCl. Considerable portions of the radioactivity were transferred to the acid fractions (A_4). The amounts ranged from 7.3% in urine of rabbits to 14.3% in urine of mice obtained after oral administration, and from 5.1% in urine of

dogs to 11.8% in urine of mice obtained after dermal application. Most of the radioactivity, however, remained in ether (E_4). These averaged 24.6 to 37.8% of the activity in urine after oral administration and 17.7 to 27.5% in urine after dermal application. The aqueous solutions (A_4) were made alkaline (pH 9) and again extracted with ether. Only small portions (1.0 to 6.2%) of the metabolites were extractable in ether (E_5). Most of the radioactivity (3.9 to 10.3%) remained in the aqueous solutions (A_5). The radioactivity in E_4 fractions was subjected to additional extraction with 0.5 N NaOH. Large portions of the metabolites were removed into the alkaline solution (A_6) ranging from 16.7 to 31.8% (oral) to 13.3 to 22.6% (dermal). Smaller amounts remained in the ether fractions (E_6) ranging from 6.7 to 15.1% and 2.2 to 11.3% in urine obtained after oral and dermal administration, respectively.

4. Thin-layer chromatography: Nine TLC solvent systems were used to achieve separation of TNT and some potential metabolites. The R_f values of these compounds in some of the systems are shown in Table 22. Only solvents I, II, V, VII, and IX were found satisfactory, although no one solvent alone could completely resolve the available potential metabolites. The polar solvent system I was advantageous for separation of trinitrobenzoic acid and the diamino derivatives, which have low R_f values with the other solvents. TNT and the other potential metabolites showed better separation with the less polar solvents II, V, VII, and IX. Solvent systems I and VII were chosen for routine analysis. Later, solvent VII was replaced by solvent IX, which contains toluene instead of benzene.

a. Pilot studies: Pilot TLC studies were performed on ethyl acetate extracts from urine samples of rats, rabbits, and dogs treated orally with ^{14}C-TNT. The TLC profiles of these extracts are shown in Figures 6 (rats), 7 (rabbits) and 8 (dogs). The use of spray reagents coupled with the R_f values helped in the detection of certain metabolic products, e.g., the amines and hydroxylamines. Rat urine extracts demonstrated a complex metabolic pattern (Figure 6), and many of the metabolites were not identified. The presence of large amounts of diamines (more of 4,6-diamino and less of 2,6-diamino derivatives) and monoamines (2-amino and/or 4-amino) was confirmed with the positive reaction to Bratton-Marshall reagent. Areas on the plates other than those of the diamines and monoamines also responded positively to the reagent, but the identities of products located in these areas are not known. A feeble reaction with the TTC spray reagent at the R_f value of the 4-hydroxylamino suggested its presence in small amounts. The TLC profiles also suggested the presence of the alcohol, the acid, minute amounts of the azoxytoluene, and the parent compound (TNT).

The TLC profile of rabbit urine (Figure 7) indicated the presence of several metabolic products. Compared to rats, larger amounts of the two monoamines were present in rabbit urine. Their presence, as well as that of the diamines, was confirmed by a positive Bratton-Marshall reaction. The presence of the 4-hydroxylamines as well as small amounts of the 2-hydroxylamines was suggested by their position (R_f) on the TLC plates and by a positive red color after spraying with TTC reagent. As indicated earlier for the rat, the presence of the acid, the alcohol, and possibly minute quantities of the azoxytoluene and TNT was suggested by their positions on the TLC plates.

Dog urine (Figure 8) contained a large amount of the 4,6-diamine and less of the 2,6-diamine and the monoamine derivatives. The TLC profiles also suggested the presence of the acid, the alcohol, and minute amounts of the 4-hydroxylamine; the latter was indicated by the feeble reaction with TTC and Benedict's reagents.

Urine samples from rats, rabbits, and dogs were hydrolyzed with β-glucuronidase, and the mixtures were extracted with ethyl acetate. Figure 9 shows the TLC of the ethyl acetate extracts of rat urine incubated with either acetate buffer (Figure 9a) or the buffer plus β-glucuronidase (Figure 9b). The only difference between the urinary profiles of both extracts is the presence of a stronger peak at R_f value of about 0.19 after incubation with β-glucuronidase (Figure 9b, solvent VII). This peak corresponds to the 4,6-diamine reference metabolite. The profile of metabolites in hydrolyzed rabbit urine showed only slight quantitative differences from the nonhydrolyzed urine (Figure 10), whereas profiles in dog urine were the same after incubation with or without β-glucuronidase (Figure 11). Among species, major quantitative and probably qualitative differences occurred between urine profiles of rabbits on the one hand and dogs and rats on the other.

b. Definitive studies: TLC studies were performed on samples of raw urine, lyophilized urine, and extracts of urine and bile obtained from different species. The TLC plates were developed with either the polar solvent I or the less polar solvent IX. Radioactivity on the plates was processed by a computer program developed in our laboratory to obtain the profiles described below.

Figure 12 shows the TLC profiles obtained from raw urine of rats and mice treated orally, dermally, or intratracheally with ^{14}C-TNT. Urine of male rats showed the presence of several metabolites (Figure 12a), most of which were more polar than TNT, but a few which were less polar. Only small portions of the radioactivity developed with the less polar solvent IX. Urine from female rats (Figure 12b) behaved similarly except that larger amounts remained at the origin after developing with solvent I. Urine obtained from dermally treated male rats (Figures 12c and d) demonstrated the presence of some TNT and/or tetranitroazoxytoluene. Some TNT and/or tetranitroazoxytoluene were also noted in the 4-hr urine obtained after oral treatment of male rats with TNT (Figure 12e). The profiles of 24-hr (Figure 12a) and 4-hr (Figure 12e) urine after oral dosing were qualitatively similar. However, there appeared to be some differences between the metabolic profiles of 4-hr urine obtained from orally (Figure 12e) and intratracheally (Figure 12f) treated rats. Male mice treated orally or dermally showed similar profiles (Figures 12g and h), which were different, at least quantitatively, from profiles obtained from rat urine (Figure 12a). Peaks at the origin in solvent I were stronger in the profiles for mice. Identity of any of the metabolites cannot be suggested from these profiles since most of the radioactivity remained at the origin in the less polar solvent IX. However, the strong positive reactions which developed after spraying with Bratton-Marshall reagent indicated the presence of mono- and diamines among the metabolites excreted from rats and mice after oral and dermal treatment with TNT. No clear positive test was indicated after spraying the plates with a solution of TTC in sodium hydroxide, which detects the hydroxylamines.

The TLC profiles of lyophilized urine obtained from rats, mice, or rabbits are shown in Figure 13. Compared to raw urine, radioactivity in lyophilized urine demonstrated more tendency to migrate in both solvents. No radioactivity remained at the origin in solvent I. Urinary profiles of male rats (Figure 13a) and female rats (Figure 13b) treated orally were qualitatively similar. Urine from dermally treated rats showed some qualitative and quantitative differences. The presence of peaks corresponding to the parent compound (TNT) and the azoxytoluene was more apparent in dermally treated animals (Figures 13c and d). Urine obtained from male mice showed the presence of several metabolites (Figure 13e). A medium-sized peak between R_f 0.4 and 0.5 was composed of mostly the monoamines (as indicated by a positive reaction with Bratton-Marshall reagent), some hydroxylamines (a feeble red color after TTC), and probably some benzyl alcohol derivative. Urine from dermally treated mice showed stronger peaks corresponding to TNT and the azoxytoluene (Figure 13f). Rabbit urine obtained after both oral and dermal treatment (Figures 13g and h) demonstrated strong peaks corresponding to the R_f values of the diamines (4,6- and 2,6-diaminotoluenes) which gave positive reactions with Bratton-Marshall reagent. In the less polar solvent IX, only small amounts of the monoamines (4-amino and 2-amino-dinitrotoluene) were present. Small amounts of the 4-hydroxylamine derivative were also demonstrated in rabbit urine, but little, if any, of the azoxytoluene was present.

In another experiment, lyophilized urine from the four different species was processed by TLC, and the plates were cut into 0.5-cm zones (Figure 14). Migration of metabolites from the origin of the plates was demonstrated only with solvent I; most of the radioactivity remained at the origin with the less polar solvent IX. Urine from male rats (Figures 14a and c) seemed to have similar profiles to that from females (Figures 14b and d), whether dosing was oral or dermal. Urine from mice (Figures 14e and f) also showed similar profiles after oral and dermal treatment. The strong peak at R_f 0.34 of rabbit urine (Figure 14g) appears to be an artifact. Some differences were demonstrated in the profiles of urine obtained from dogs after oral and dermal dosing (Figures 14k and l). With solvent I, most radioactivity remained near the origin after oral administration (Figure 14k); after dermal application the radioactivity migrated readily (Figure 14l).

Figure 15 shows the TLC of the ethyl acetate-extractable material obtained from rat urine incubated with water, β-glucuronidase, or aryl sulfatase. With the more polar solvent I, all the activity as well as the reference metabolites migrated from the origin of the plates. Several metabolites could be demonstrated after developing with both solvents I and IX. The presence of the monoamines (4-amino and/or 2-amino derivatives) was clearly demonstrated (Figure 15a). Only small amounts of the diamino derivatives were detected. The presence of small amounts of the 4- and 2-hydroxylamines was suggested by the feeble reactions obtained after spraying with TTC or with Benedict's reagent. Although incubation with β-glucuronidase increased considerably the amounts of radioactivity extractable in ethyl acetate, there was no apparent change in TLC profiles with both solvents (Figure 15b). On the other hand, incubation with aryl sulfatase caused no change in the amounts of ethyl acetate-extractable radioactivity but seemed

to alter the metabolic profiles of both solvents (Figure 15c). There seemed to be a considerable increase in the peaks which corresponded to the diamino derivatives. TLC profiles of urine from female rats without or with enzyme hydrolysis were similar to those of male rats (Figures 15d, e, and f).

The TLC profiles of the ethyl acetate-extractable material from urine of male rats treated orally or dermally with ^{14}C-TNT are compared in Figure 16. After oral administration, these metabolites in urine migrated readily in solvent I (Figures 16a, e, and k). However, most of the metabolites were highly polar and remained at the origin in the less polar solvent IX. The presence of diamino and monoamino derivatives was readily demonstrated. Quantitative determination was not possible. Positive tests to TTC and Benedict's reagents suggested the presence of small amounts of the hydroxylamines. However, no azoxytoluene or TNT was demonstrated. From the TLC profiles, it appeared that the alcohol and the acid were present in small quantities. After hydrolysis with β-glucuronidase, only slight differences in the metabolic profiles were noted (Figures 16b, f, and l). Less radioactivity was recovered at the origin in solvent IX. Still, however, it constituted 65% of the activity applied on the plate. Urine obtained from rats treated dermally behaved similarly (Figures 16c, d, g, h, m, and n). Although the extracted radioactivity was higher after β-glucuronidase hyrolysis, the metabolic profiles did not change considerably, and a major portion of the activity still remained at the origins of the plates with solvent IX. When urine samples from different rats treated orally or dermally were compared, only slight quantitative differences seemed to be demonstrated. Some urine samples from dermally treated rats showed higher excretion of the parent compound, TNT.

The profiles of urinary metabolites obtained from female rats are shown in Figure 17. No apparent qualitative differences were noted between urine profiles of males and females. As noted in males, urine from dermally treated female rats showed slightly higher excretion of the parent compound, TNT.

The ethyl acetate extracts obtained from the 4-hr urine of male rats are shown in Figure 18. These rats were treated with ^{14}C-TNT orally or intratracheally. Bile was collected at the same time through a biliary cannula. Qualitatively, the metabolic profiles of these urine samples showed similarity to those of the 24-hr urine (Figure 16). Quantitatively, however, stronger peaks in the area of R_f 0.4 to 0.6 (solvent I) were present in the 4-hr urine. Hydrolysis with β-glucuronidase increased the extractable radioactivity but had no apparent effect on the pattern of metabolites (Figures 18a and b). Urine profiles from intratracheally treated rats showed some differences from urine profiles after oral dosing (Figures 18c and d). The presence of the diamines, the monoamines, and small amounts of the hydroxylamines was confirmed by the positive response to the chemical spray reagents.

The TLC profiles of 4-hr urine obtained from female rats treated orally or intratracheally are shown in Figure 19. As noted in urine of male rats, the metabolic pattern in solvent I was different from that obtained for the 24-hr urine samples (Figure 16). The TLC profiles obtained

from urine of orally treated animals (Figures 19a and b) were distinctly different from those of urine obtained after intratracheal dosing (Figures 19c and d). This difference was noted in both solvents I and IX.

Figure 20 shows the TLC profiles of the ethyl acetate-extractable material obtained from urine of male mice. Profiles in both solvents I and IX indicate that extensive metabolism of TNT occurred in mice. Hydrolysis with β-glucuronidase (Figures 20b, d, f, h, k, and l) did not cause any major changes in the metabolic patterns as compared with those without hydrolysis (Figures 20a, c, e, and g). Urine from dermally treated mice (Figures 20a, b, e, f, and l) was not qualitatively different from that obtained after oral dosing (Figures 20c, d, g, h, and k). Major differences between the urine from mice (Figure 20) and rats (Figure 16) were the presence of less polar metabolites at the origin of the TLC plates for mice, fewer diamino derivatives, but more of the monoamines, hydroxylamines, and probably the alcohol.

The urinary TLC profiles of the ethyl acetate extracts obtained from male rabbits treated with TNT are shown in Figure 21. As was noted for rat and mouse urine, rabbit urine without hydrolysis contained several metabolic products (Figures 21a, c, e, g, k, and m), many of which were highly polar. Quantitatively, rabbit urine contained larger amounts of the monoamines than did mouse urine. The 2,6- and/or 2,4-diamino derivatives were also present. Positive tests with TTC and Benedict's reagents confirmed the presence of the 4-hydroxylamine and to a lesser extent the 2-hydroxylamine. TLC profiles also suggested the presence of the alcohol and the acid, but this could not be confirmed. Of significance, TNT and the azoxytoluene were absent. In earlier studies, the hydroxylamines were found to decompose during the extraction processes leading to the azoxytoluene. The same metabolic profiles were obtained after incubation of rabbit urine with β-glucuronidase (Figures 21b, d, f, h, l, n, o, and p). In addition, profiles obtained from urine of dermally treated rabbits (Figures 21c, d, g, h, m, n, and p) were similar to those of orally treated rabbits (Figures 21a, b, e, f, k, l, and o), although smaller amounts of the acid seemed to be present in urine after dermal treatment.

The TLC profiles of urine samples obtained from male dogs treated orally or dermally are shown in Figure 22. The metabolic profiles of urine from dogs were qualitatively similar to those obtained from rats and mice. The presence of the monoamines, diamines, and hydroxylamines was confirmed by the positive reactions with Bratton-Marshall, TTC, and Benedict's reagents. No apparent differences were observed in the metabolic patterns of urine incubated with water or with β-glucuronidase. Urine from dermally treated dogs (Figures 21c, d, g, h, and l) appeared to contain less polar material at the origin of solvent IX and less acid as shown in solvent I.

The aqueous nonextractable material remaining after ethyl acetate extraction of rat, rabbit, and dog urine was evaporated under N_2, then subjected to TLC analysis using the two solvent systems I and IX. Although some of the radioactivity migrated from the origin in solvent I, most remained at the origin in both solvents I and IX (Figure 23). Reaction with

Bratton-Marshall reagent suggested that only minute amounts of free amines were present in these aqueous fractions. Positive response to the reagent was detected in the areas corresponding to the diamines. These diamines are basic and are expected to remain in the aqueous phases after acidification with hydrochloric acid. Some, however, were detected in ethyl acetate extracts. No hydroxylamines were detected after spraying the plates with TTC or Benedict's reagents.

Figure 24 shows the TLC profiles of TNT metabolites in bile of rabbits and dogs. These bile samples were extracted with ethyl acetate after acidification with dilute hydrochloric acid. The extracted bile samples migrated readily with solvent I but to a lesser extent with solvent IX. The monoamines and, to a lesser extent, the diamines and hydroxylamines, were detected in rabbit bile (Figure 24a). Minute amounts of TNT were also present. In dog bile (Figure 24b), there was less radioactivity remaining at the origin of solvent I than in rabbit bile. The monoamines, diamines, and hydroxylamines were detected in dog bile obtained after oral treatment with TNT. The presence of the acid, alcohol, and TNT was suggested from their migration alongside the authentic metabolites. Hydrolysis with β-glucuronidase did not markedly alter the metabolic profiles of dog bile (Figure 24c). After dermal application of TNT, dog bile contained fewer polar metabolites and more of the parent compound, TNT (Figures 24d and e). The aqueous extracts obtained from dog bile (Figure 21f) contained highly polar metabolites. Except for the presence of some diamines (positive with Bratton-Marshall reagent), most of these metabolites were not identified.

c. Fractionated urinary metabolites: Urine samples from rats, mice, rabbits, and dogs were extracted with ether at different pH conditions in order to fractionate the urinary products into subgroups according to their neutral, acidic, or basic characteristics (see Figure 5a). The ether extracts (E_1-E_6) were evaporated and subjected to TLC analysis. A parallel experiment was performed in which TNT and nine potential metabolites were fractionated between the organic and aqueous phases. Recoveries of various compounds in the ether extracts were described in detail in an earlier section and are summarized as follows: E_1 contained large amounts of TNT, some of the monoamines, hydroxylamines, trinitrobenzyl alcohol, and azoxytoluene, and small amounts of the diamines. E_2 contained all the trinitrobenzoic acid, most of the monoamines, hydroxylamines, alcohol, and azoxytoluene, and some of the diamines and TNT. The basic fraction E_3 had most of the diamines and some of the monoamines. The E_2 fraction was subfractionated into E_4, which contained all the trinitrobenzoic acid, most of the hydroxylamines, trinitrobenzyl alcohol, and azoxytoluene, and some TNT; and E_5, which had most of the monoamines and some diamines. Subfraction E_6, derived from the E_4 fraction, contained most of the alcohol and azoxytoluene and some hydroxylamines.

The percentage of extractable radioactivity in different fractions of urines from different species and the TLC profiles of these different fractions are illustrated in Figures 25 through 40. Some of these profiles (e.g., E_3, E_5, and E_6) are simple and contain only a few major peaks, but others (e.g., E_1, E_2, and E_4) demonstrate complex patterns

of radioactive peaks. In almost every fraction, several metabolites of unknown identity were separated along with the anticipated products. Occasionally, a known metabolite was recovered in more than one fraction, and solubility characteristics seemed to have been altered in the presence of other metabolic products.

Figure 25 indicates the percentage of extractable radioactivity in different fractions of urine obtained from rats treated orally with TNT. The TLC profiles of the various fractions are shown in Figure 26. Fraction E_1 contained small amounts of the alcohol, monoamines, hydroxylamines, and diamines but may also have had some of the acid and probably the azoxytoluene and/or TNT. Most of the radioactivity was contained in highly polar material which did not migrate with the less polar solvent IX. Fraction E_2 was qualitatively similar to E_1, but it contained larger amounts of trinitrobenzoic acid and dinitrotoluenes. Also, major portions of the radioactivity remained at the origin when developed with solvent IX. In addition to the monoamines and diamines, fraction E_3 contained small amounts of hydroxylamines and the azoxytoluene but also other unidentified polar material. Fraction E_4 demonstrated a complex profile which contained large amounts of the trinitrobenzoic acid and lesser amounts of trinitrobenzyl alcohol, hydroxylamines, and TNT. In addition to some unidentified polar products, the basic fraction E_5 demonstrated large amounts of the monoamines, diamines, other unidentified amino derivatives (positive with Bratton-Marshall reagent), and small quantities of the azoxytoluene. E_6 contained large amounts of the alcohol and small quantities of azoxytoluene and hydroxylamines in addition to other unidentified metabolites. Most of the polar metabolites demonstrated in fraction E_4 were removed by sodium hydroxide and were absent from E_6.

The amount of extractable radioactivity from urine of dermally treated rats is illustrated in Figure 27. TLC profiles are shown in Figure 28. These profiles are similar to those obtained from urine of orally treated rats (Figure 26) with only a few exceptions. E_1 contained larger amounts of the parent compound, TNT, and/or the azoxytoluene. Fraction E_3 demonstrated the presence of less monoamines and more unidentified highly polar metabolites. On the other hand, more of the monoamines and fewer of the polar products were present in fraction E_5. E_6 contained appreciable amounts of TNT and/or the azoxytoluene.

Figure 29 summarizes the extractable radioactivity in different fractions of urine obtained from mice treated orally with ^{14}C-TNT. The TLC profiles of the various fractions are shown in Figure 30. Fraction E_1 contained relatively large amounts of the monoamines and lesser quantities of the diamines, hydroxylamines, the alcohol, and the parent compound, TNT. Major differences between the profile of this fraction in mice (Figure 30) and rats (Figure 26) are the presence in mice of larger amounts of the monoamine and small amounts of the highly polar unidentified metabolites remaining at the origin after developing with solvent IX. E_2 demonstrated the presence of large amounts of trinitrobenzoic acid, some of the monoamines, diamines, hydroxylamines, and the alcohol. It also contained large quantities of unidentified polar products. The fraction E_3 contained mainly the

monoamines, some of the diamines, and some basic polar metabolites which reacted positively with Bratton-Marshall reagent. E_4 showed the presence of the acid, the alcohol, and some hydroxylamines and TNT. The E_5 fraction was spilled before analysis with TLC. E_6 contained mostly the alcohol, some hydroxylamines, and large amounts of TNT and/or the azoxytoluene. The latter is the likely possibility since a strong peak at this position was not demonstrated in fractions E_2 and E_4. It is probably formed from the hydroxylamines during the extraction of E_4 with sodium hydroxide.

The amounts of extractable radioactivity from urine of dermally treated mice are illustrated in Figure 31. TLC profiles are shown in Figure 32. These profiles are similar to those obtained from urine of orally treated mice (Figure 30), with the exception that E_1 and E_4 contained larger proportions of TNT and E_6 demonstrated stronger peaks corresponding to TNT and/or the azoxytoluene.

The extractable radioactivity in different fractions of urine obtained from rabbits treated orally with ^{14}C-TNT is shown in Figure 33. The TLC profiles of the various fractions are illustrated in Figure 34. E_1 contained several metabolites which included varying amounts of the monoamines, hydroxylamines, alcohol, and some diamines. The absence of TNT and the azoxytoluene was demonstrated. A major portion of the radioactivity was contained in unidentified polar products. Fraction E_2 contained large amounts of the acid, some monoamines, hydroxylamines, diamines, and probably the alcohol. Only trace amounts of TNT and/or the azoxytoluene were present. E_3 contained mainly the monoamines and azoxytoluene and smaller quantities of the diamines. Large amounts of the acid, alcohol, and hydroxylamines and smaller quantities of the azoxytoluene were demonstrated in E_4. Fraction E_5 contained primarily the monoamines and diamines and other unidentified amino derivatives. The largest portion of E_6 is probably the alcohol. It also contained large amounts of the azoxytoluene and small proportions of the hydroxylamines. The azoxytoluene seemed to have been formed during the sodium hydroxide extraction of E_4.

The amounts of extractable radioactivity from urine of dermally treated rabbits are illustrated in Figure 35. TLC profiles are shown in Figure 36. The major difference between these profiles and those of urine obtained from orally treated rabbits (Figure 34) was in fraction E_1. Fraction E_1 from urine of dermally treated rabbits demonstrated increased amounts of the monoamines, hydroxylamines, alcohol, and azoxytoluene and a sharp decrease in the amounts of polar metabolites not migrating with solvent IX.

Figure 37 indicates the extractable radioactivity in different fractions of urine obtained from dogs treated orally with ^{14}C-TNT. The TLC profiles of the various fractions are illustrated in Figure 38. Fraction E_1 contained several metabolic products including the monoamines, hydroxylamines, some diamines, and probably the alcohol. No TNT or azoxytoluene was demonstrated. The complex metabolic profile of E_2 contained the acid, monoamines, diamines, hydroxylamines, some TNT, and probably the trinitrobenzyl alcohol. E_3 contained mainly the monoamines, diamines, some TNT and/or azoxytoluene, and unidentified polar products. Fraction E_4 contained large amounts of the acid and some hydroxylamines, TNT, and probably the alcohol.

Large amounts of monoamines, the azoxytoluene and/or TNT were present in fraction E_5. Fraction E_6 contained large amounts of the alcohol and the parent compound, TNT, and small amounts of the hydroxylamines and the azoxytoluene. The latter appeared to be formed during the extraction of E_4 with sodium hydroxide.

The amounts of extractable radioactivity from urine of dermally treated dogs are illustrated in Figure 39. TLC profiles are shown in Figure 40. These profiles were similar to those obtained from urine of orally treated dogs (Figure 37) with only few exceptions. E_1 contained considerable amounts of TNT, which was absent from urine obtained after oral treatment. Fraction E_3 contained fewer monoamines and more of the diamines and unidentified polar metabolites. E_6 showed the presence of larger amounts of the parent compound, TNT, and the azoxytoluene.

5. Gas-liquid chromatography: Retention times of TNT and the available potential metabolites of TNT were determined as described in Section A, "Methods." The retention times for an isothermal (170°C) elution of TNT and potential metabolites are shown in Table 23. Attempts to achieve adequate separation of a mixture of TNT and the potential metabolites on either column were unsuccessful even when temperature programming was utilized.

6. High performance liquid chromatography: Different HPLC systems were tested for the separation of TNT and some potential metabolites. The first system used (system 1) was normal phase chromatography. It gave adequate separation of these compounds, but it was not adequate for the separation of more polar metabolites. Therefore, three other systems were examined which utilized counter-ion reverse phase chromatography. The retention times of TNT and some potential metabolites in this system are shown in Table 24. System 4 appeared to give good separation and the best defined peaks. This system was selected for the analysis of TNT and its metabolites in rat urine.

Figure 41 illustrates the chromatographic profile of raw urine obtained from rats treated orally with ^{14}C-TNT. Some minor peaks were observed with retention times corresponding to those of TNT, the diamines, and the alcohol. However, most of the radioactivity in urine was eluted in adjacent fractions with similar retention times. Although some of these fractions have the same retention times as the 2-amino, 4-amino, and 2-hydroxylamino derivatives, confirmation of the presence of these metabolites was not possible. HPLC analysis was also performed on samples of rat urine hydrolyzed with β-glucuronidase. Although there were some apparent differences in the metabolic profiles after hydrolysis with β-glucuronidase (Figure 42), the identity of these metabolites was not confirmed. Better resolution of the metabolites in rat urine was obtained when smaller fractions of the eluted products were collected (Figure 43). However, the metabolic profile was also more complex. Since the use of HPLC offered no major advantage over TLC for the analysis of TNT profiles in urine of different species, its use was discontinued.

C. Discussion

1. Potential metabolites of TNT: Because of the presence of four functional groups on the TNT molecule, a variety of metabolic products could be formed. These may result from oxidation of the methyl group to alcohol, aldehyde, or acid; oxidation of the benzene nucleus to phenols; reduction of one or more of the nitro groups to hydroxylamino or amino compounds with the possibility of coupling of some of these metabolites; and conjugation of one or more of the resulting products (alcohols, acids, amines, hydroxylamines, etc.) to yield glucuronides, ethereal sulfates, substituted hippuric acid, or glutathione conjugates. Simultaneous oxidation and reduction followed by conjugation is also a possibility. These hypothetical pathways, which are shown in Figure 1, illustrate the complexity of the metabolic behavior of TNT. The problem of metabolite identification is complicated by the similar solubility characteristics possessed by these compounds of such closely related chemical structure.

Earlier studies by Voegtlin et al.[42] and Dale[57] have suggested that the reduction products, 4-amino-2,6-dinitrotoluene and 2,6,2',6'-tetranitro-4,4'-azoxytoluene, are excreted in the urine of workers exposed to TNT. Reduction of a single nitro group of TNT was shown to occur also in rabbits, leading to the formation of 4-amino- and 6-amino-dinitrotoluenes.[56] Channon et al.[56] postulated that the first step in the reduction of the nitro group is the production of a hydroxylamine derivative. They isolated 4-hydroxylamino-2,6-dinitrotoluene as an aldoxime after reaction with benzaldehyde, but they failed to isolate its isomer, 2-hydroxylamino-4,6-dinitrotoluene. However, the isolation of the reduction product, 2-amino-4,6-dinitrotoluene, led to the conclusion that the 2-hydroxylamine is a step in its formation.

Because Wyon found the hydroxylamine derivative to be more toxic than the parent TNT, the isolation of hydroxylamine is of interest.[59] The hydroxylamine is a powerful methemoglobin producer in vitro, while TNT itself is only a weak producer of methemoglobin.[59] In addition, the formation of hydroxylamines is implicated in the carcinogenic responses induced by several carcinogenic amino and nitro compounds.[60] In Channon et al. studies, only 1% of the administered TNT dose was accounted for as hydroxylamine.[56] This, however, seems to be less than the actual amount present because of the great ease of conversion to the azoxy derivative.

Oxidation of TNT may result in the formation of alcohol or acid. These oxidation processes are hypothetical and are based on some indirect evidence obtained from some early studies by Channon et al.[56] Rabbits excreted 48% of the administered TNT dose as glucuronides, which were believed to arise from oxidation products of TNT such as trinitrobenzyl alcohol. The possibility of glucuronide conjugation with the amino or hydroxylamino derivatives was not considered. Also, the suggestion by Lemberg and Callaghan[55] that nitrophenylenediamine is excreted in rat urine indicates that this oxidative pathway may be operative. Williams[61] suggested that the loss of the methyl group could probably occur by oxidation of TNT to the alcohol, then the acid, followed by decarboxylation and reduction of the nitro group.

Amino-nitrocresol is another oxidation product whose presence in rat urine was suggested. The mechanism of its formation is not known.

In vitro experiments suggested that the liver is the major site for TNT biotransformation.[62] Studies using liver, muscle, and heart preparations showed that TNT was reduced by liver homogenates to 4-amino-2,6-dinitrotoluene. The rate of reduction was more rapid under anaerobic conditions. TNT metabolism occurred in a system containing reduced nicotinamide adenine dinucleotide (NADH) and a purified flavoprotein. It was also suggested that TNT was reduced to hydroxylamines by xanthine oxidase.

2. Extraction procedures: Since the beginning of this century, extensive work has been carried out to isolate and identify TNT metabolites in animals,[56-58] and humans.[55,57] Only limited success was achieved because of the difficulties encountered during the isolation procedures. Low recovery was encountered when urine samples were extracted with ether. It was found[56] that ether extracted little TNT-derived material from urine until it was acidified. Even after acidification, no more than 15% of the dose administered to rabbits was excreted as compounds soluble in ether. In the present study, the use of ether under mildly acidic conditions resulted in higher recoveries. This was further increased by extracting the urine with ethyl acetate under the same acidic conditions. The use of strong acid or base was avoided since this would undoubtedly cause alterations of the metabolites during the extraction process. 2,6,2',6'-Tetranitro-4,4'-azoxytoluene, which was reported as one of the TNT metabolites in rabbit and human urine,[57] was found later to be an artifact that was formed from the 4-hydroxylamine under the conditions of the isolation procedure. This azoxytoluene was shown to be absent from freshly voided urine of rabbits given TNT.[56] Alterations of TNT metabolites could also occur during storage. In our laboratory, the trinitrobenzyl alcohol and the trinitrobenzoic acid, two potential metabolites of TNT, were shown by TLC analysis to decompose to several products when stored in methanolic solutions.

Several methods were used in the present study to separate the metabolic products of TNT from urine and bile. Attempts to purify urine samples by Amberlite resin were not successful. Direct analysis of the raw or lyophilized urine was not successful, and separation of the highly polar and complex mixture of metabolic products proved difficult even with HPLC analysis. A more useful approach was the extraction of metabolic products into organic solvents. Acidification of the urine before extraction proved essential. Since these extracts still demonstrated complex metabolic profiles, a method was developed to fractionate the radioactivity in urine samples into subgroups according to their solubilities in the ether or aqueous extracts under different pH conditions. A mixture of TNT and nine potential metabolites was processed similarly and fractionated according to the neutral, acidic, or basic characteristics of each compound. Although this fractionation technique was successful when used with this mixture, it showed only limited success when urine samples were processed similarly. In almost every fraction, several metabolites of unknown identity were separated along with the anticipated products. Occasionally, a known metabolite was recovered in more than one fraction, and solubility characteristics seemed to have been altered in the presence of other metabolic products in the urine samples.

3. Separation procedures: Analysis of TNT metabolic profiles in urine and occasionally in bile was carried out by TLC. The use of GLC was discontinued since it was not possible to achieve a good separation of TNT and some potential metabolites. In view of the demonstrated high polarity of the excretory products, HPLC analysis was attempted. It offered no major advantage, however, over the use of TLC in this study since the major portion of the radioactivity excreted in urine was eluted in adjacent fractions. Although a good separation of synthetic mixtures was achieved by HPLC, poor separation occurred when urine samples were processed by this method. Studies to analyze the metabolic profiles of TNT in different species and after different routes of administration were, therefore, continued with TLC using two solvent systems with different polarity. TLC analysis had been useful in comparing the metabolic patterns of TNT metabolites. However, it required reference standards of potential metabolites for comparison, and many of these were not available commercially and could not be prepared in pure form in the MRI laboratories. Urine and bile contained large numbers of metabolic products. Attempts to isolate some of these metabolites in pure form by preparative TLC met with only limited success. The metabolites were assigned tentative identification based on comparing R_f values with those of some potential metabolites that were available and based on their solubility characteristics and reactions with certain specific chemical reagents. Because of the complexity of metabolic profiles, quantitative determinations were not possible.

4. Metabolic profiles: Early studies have suggested that urine from TNT workers contained the same metabolites reported in rabbit urine, namely 4-hydroxylamino-2,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, and 2-amino-4,6-dinitrotoluene.[56] Rat urine contained, in addition to the monoamines, 2,4-diamino-6-nitrotoluene and probably 5-nitrophenylenediamine.[55] In the urine of dogs which received TNT orally, Snyder[58] was unable to demonstrate the presence of TNT, its oxidation products (alcohol, aldehyde, or acid), or its reduction products (diamino and triaminotoluenes). In the present studies, TNT profiles indicated that extensive biotransformation of TNT occurred in all species examined.

a. Rats: Metabolic profiles of rat urine demonstrated the presence of appreciable amounts of amino products (positive with Bratton-Marshall), some of which were not identified. Large quantities of diamines (more of 4,6-diamino derivative) and monoamines (the 4-amino and/or 2-amino) were present. Rat urine also contained small amounts of the 4-hydroxylamine and, to a lesser extent, the 2-hydroxylamine. Their presence was only demonstrated after ethyl acetate and/or ether extractions. Positive reactions with Benedict's reagent and the more specific triphenyltetrazolium chloride reagent indicated the presence of both hydroxylamines at the R_f positions of the corresponding standards.

The presence of appreciable amounts of trinitrobenzyl alcohol and trinitrobenzoic acid in rat urine was suggested by their R_f values. The latter compound was also indicated by its solubility behavior during the fractionation of urine. The absolute identity of both compounds, however, could not be confirmed.

Small amounts of azoxytoluene were detected in the TLC profiles of fractionated rat urine. The azoxytoluene was also found in urine of mice, dogs, and, to a greater extent, rabbits, mostly in the E_6 fraction after sodium hydroxide extraction of E_4. The azoxytoluene was probably formed from the hydroxylamines during the extraction process in the presence of alkalis.

Metabolic profiles of urine from male and female rats showed no significant differences. The urine collected from bile duct-cannulated rats showed metabolic profiles which differed, at least quantitatively, from that of the urine collected from noncannulated rats. It contained more polar metabolites and had more TNT and probably the azoxy compound. A quantitative difference was noted in the metabolic profiles of urines collected from orally versus intratracheally treated rats. On the other hand, the differences between urine collected from orally and dermally treated rats were minimal; more TNT was eliminated after dermal application.

Metabolism of TNT by the intestine or intestinal microflora was not examined in the present study. Others have demonstrated that the nitro group is highly susceptible to reduction by intestinal microflora.[75] From the present studies, it appears that TNT reduction occurred primarily in the liver. Bile and urine collected from biliary cannulated rats contained large amounts of reduced TNT metabolites, as indicated by the R_f values and the positive reactions with Bratton-Marshall, triphenyltetrazolium chloride and Benedict's reagents. This, however, does not rule out further intestinal metabolism occurring after excretion of the metabolic products through bile.

The nature of the red pigment excreted after TNT intake was examined by Channon et al.[56] They suggested that this pigment, which did not appear to account for a significant amount of metabolites, might be a partial reduction product of 2,4,6-trinitrobenzyl alcohol. They also suggested that the red pigment might be a salt of TNT or one of its metabolites since it decolorized on acidification with mineral acids. In the present study, rat urine was bright red in color even though urine is slightly acidic. On the other hand, rabbit urine is alkaline, but the red color was not apparent.

b. Mice: Mice also excreted monoamines, diamines, and small amounts of the hydroxylamines. The presence of azoxytoluene was demonstrated only after fractionation of urine samples under acidic and basic conditions. Considerable amounts of the benzyl alcohol and the acid seemed to be present. Metabolic profiles of urine from orally and dermally treated mice showed no major differences except for the presence of larger quantities of TNT in urine of dermally treated mice. Compared to rats, urine of mice contained lesser amounts of polar metabolites and diamines. Quantities of monoamines and hydroxylamines in urine of mice seemed to be larger than those in rats.

c. Rabbits: The metabolic profiles of rabbit urine demonstrated the presence of larger amounts of monoamines. The 4,6-diamine and, to a lesser extent, the 2,6-diamine were also present. The failure of earlier investigations to demonstrate the presence of diamines in rabbit urine

was probably due to the strong acid conditions used.[56] As noted in early studies, the 4-hydroxylamine was present in appreciable quantities in rabbit urine. The presence of smaller amounts of the 2-hydroxylamine was also suggested by comparison with the R_f value and behavior of an authentic sample. Urinary profiles of rabbit urine seem to contain trinitrobenzyl alcohol and trinitrobenzoic acid, as indicated by their R_f positions. With the mild extraction procedure used in this study, no azoxytoluene was demonstrated. However, after fractionation with ether in the presence of alkali, TLC peaks corresponding to azoxytoluene were found. Of significance was the absence of TNT from the urinary profiles of rabbits. After hydrolysis with β-glucuronidase, the urinary profiles remained the same. Urine obtained from dermally treated rabbits showed a sharp decrease in polar metabolites, including acid, and some increases in monoamines, hydroxylamines, and the azoxytoluene.

d. Dogs: The metabolic profiles of dog urine contained large amounts of the 4,6-diamines and the 2,6-diamines. Appreciable quantities of the monoamines (2- or 4-substituted) and probably the alcohol and acid were also present. The presence of small amounts of the 4-hydroxylamine and, to a lesser extent, the 2-hydroxylamine was indicated by comparing their migration and chemical behavior with authentic samples. The minute amounts of azoxytoluene present seemed to be formed during the extraction process. As shown in the urine of other species, β-glucuronidase hydrolysis caused no apparent differences in metabolic profiles. Urine obtained after dermal application contained smaller amounts of polar metabolites and larger amounts of the parent compound TNT as compared to urine from orally treated dogs.

5. Metabolite conjugates: Glucuronide conjugation appears to play an important role in the metabolism of TNT. Other conjugates and probably inorganic salts may also be present. Channon et al.[56] found that, even after acidification of rabbit urine, no more than 15% of the administered dose was excreted as compounds soluble in ether. They suggested that the ether extracts contained metabolites excreted in an unconjugated form and possibly small amounts of acetylated amino derivatives. The remainder of the doses administered were probably eliminated as conjugates, e.g., glucuronides and sulfates. The excretion of compounds in combination with glucuronic acid was suggested based on an increase in glucuronides in urine after TNT dosing. The present study confirms these earlier findings. Based on the increase in extractable radioactivity after hydrolysis with β-glucuronide, major portions of TNT metabolites were excreted as glucuronide conjugates. The amounts of glucuronides varied among species. The least amounts occurred in urine of mice. Urine from dermally treated animals contained lesser amounts of glucuronide conjugates than did urine from orally treated animals. Amounts of glucuronide in urine from bile duct-cannulated rats were less than amounts from noncannulated rats. Bile contained large amounts of glucuronide conjugates; most compounds of low molecular weight, e.g., TNT metabolites, are excreted in bile only after conjugation with glucuronic acid or glutathione.

Although the extractable radioactivity increased considerably after hydrolysis with β-glucuronidase, major changes in the metabolic profiles after hydrolysis were not apparent. The only notable changes were increased

amounts of diamino metabolites in urine of some species, e.g., rat. On the other hand, some notable changes were demonstrated in the TLC profiles of rat urine after incubation with aryl sulfatase. Considerable increases in polar metabolites including the diamines occurred. However, there were no increases in the extractable radioactivity. This was taken as an indication of the absence of sulfate conjugates. Early studies have demonstrated no rise in ethereal sulfate excretion after administration of TNT to rabbits.[56]

VII. CONCLUSIONS AND RECOMMENDATIONS

The present studies indicate that TNT administered orally, dermally, or intratracheally was readily absorbed, distributed, metabolized, and excreted in urine and to a lesser extent in feces. Absorption by the dermal route was slower than by the oral or intratracheal routes. Species differences in dermal absorption were found; the highest absorption occurred in rabbits, followed by mice, rats and dogs. TNT was more rapidly absorbed after intratracheal instillation than after oral or dermal administration. Biliary excretion and enterohepatic circulation appeared to play an important role in the disposition and metabolism of TNT.

TNT was metabolized extensively in all species examined, whether treatment was oral, dermal, or intratracheal. Most of the metabolic products were highly polar with very low extractability in organic solvents. Large portions of these products were conjugated with glucuronic acid, but no conjugation with sulfuric acid was detected. Other conjugates or inorganic salts of TNT metabolites were probably present. Most of the metabolic products were reduction derivatives, including the hydroxylamines, the monoaminodinitro and the diaminomononitro derivatives. The benzyl alcohol and the acid seemed to be present in medium quantities, but this was not confirmed. The parent compound, TNT, was demonstrated in the urine of some species but only in minute quantities. The mild extraction procedures used in the present study minimized the alterations of the hydroxylamines to the azoxytoluene, but the latter was present, especially after fractionation of the urinary products in the presence of NaOH. Other products of TNT metabolism were not identified due to lack of authentic standards for comparison.

Rabbit urine showed a unique metabolic profile which differed quantitatively, and probably qualitatively, from the metabolic profile of rats, mice, and dogs. The presence of larger quantities of the hydroxylamines and monoamines in rabbit urine was demonstrated. Rabbit urine also contained either or both of the diamines found in the urine of other species. The metabolic profiles of urine from rats, mice, and dogs also differed quantitatively. Even within species, some quantitative differences were demonstrated between individual animals. Major quantitative differences were demonstrated in the urinary profiles of orally versus intratracheally treated rats. On the other hand, the differences between urine profiles obtained from orally and dermally treated animals were minimal; larger amounts of the parent compound, TNT, were eliminated after dermal application. Although the extractable radioactivity increased considerably after β-glucuronidase hydrolysis of urine from different species following different routes of administration, major changes in the metabolic profiles were not apparent.

The results of the present study provide some data relevant to the selection of species and routes of exposure for any subsequent chronic toxicity studies. Based on urinary excretion patterns reported herein in comparison to earlier published information on humans, the present results would suggest that the rabbit may approximate humans more closely than do mice, rats, or dogs. The rabbit certainly excretes higher levels of at

least one of the potentially more active metabolites, hydroxylamine, which might enhance any potential carcinogenic responses. The rabbit, however, is not an animal commonly used in carcinogenic bioassays and certainly is not recommended herein for that purpose. The laboratory rat historically has been used extensively in carcinogenesis studies; the metabolic profile of TNT in rats is qualitatively similar to rabbits and also to humans. As such, the rat remains the most appropriate animal model for chronic studies. Mice could also be used for carcinogenic studies, but the only apparent advantage would be in the use of larger numbers of animals. Dogs would not be suitable for carcinogenic studies if for no other reason than the time interval (8 to 10 years) that might be required to undertake a study of this nature.

Human exposure to TNT for the most part is probably through the dermal and inhalation routes. Dermal exposure in humans can probably be simulated by oral exposure since the metabolic profiles in the experimental animals were qualitatively similar following oral and dermal exposures. The major issue would be to adjust dose levels or at least to interpret experimental results as a function of absorption since the present data demonstrated less absorption of TNT following dermal application to rodents. Additional metabolism studies would be warranted to better define relative absorption following oral and dermal exposures.

The question of simulation of inhalation exposure using oral administration could not be completely resolved in the present studies. Failure to produce adequate dispersions or aerosols for inhalation exposures negates any direct resolution of the problem. Metabolic data, however, obtained following intratracheal instillation demonstrated quantitative differences in urinary profiles, hence metabolism of the compound. Moreover, absorption, distribution, and elimination were more rapid following intratracheal instillation. On this basis, use of oral exposures to simulate inhalation exposures would not appear to be appropriate. However, because of the apparent problems associated with the generation of aerosols for inhalation, oral exposures may be necessary.

The need for further research is obvious. Additional efforts should be directed to developing techniques to produce aerosols or particulate dispersions appropriate for inhalation studies. If successful, additional metabolic studies would be required to determine absorption, distribution, metabolism, and excretion of TNT following inhalation exposure. If not successful and oral exposures are used for chronic studies, additional metabolism studies would be warranted to better correlate TNT disposition and metabolism following oral exposure and intratracheal instillation on the assumption that this route would simulate absorption after inhalation exposure.

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TABLE 1

TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY 24 HR AFTER ORAL ADMINISTRATION OF ^{14}C-TNT (100 mg/kg) TO SPRAGUE-DAWLEY RATS[a]

	Males		Females	
Tissue/Excretum	Concentration (µg eq/g or ml)	Percent of Dose	Concentration (µg eq/g or ml)	Percent of Dose
Blood[b]	2.884 ± 0.676	0.200 ± 0.047	4.175 ± 0.679	0.292 ± 0.047
Liver	10.748 ± 2.559	0.385 ± 0.100	13.854 ± 3.415	0.428 ± 0.069
Kidneys	3.491 ± 0.720	0.169 ± 0.031	2.575 ± 0.731	0.245 ± 0.052
Lungs	0.299 ± 0.080	0.019 ± 0.006	0.378 ± 0.117	0.038 ± 0.010
Spleen	1.767 ± 0.423	0.002 ± 0.000	4.718 ± 3.011	0.003 ± 0.002
Brain	0.152 ± 0.067	0.008 ± 0.003	0.221 ± 0.064	0.016 ± 0.003[d]
Muscle[c]	0.777 ± 0.213	0.309 ± 0.085	2.154 ± 0.153[d]	0.863 ± 0.062[d]
GI Tract plus contents	91.217 ± 8.891	29.756 ± 2.681	99.821 ± 23.648	33.937 ± 6.456
Feces		8.050 ± 2.444		2.057 ± 0.767[d]
Urine		52.719 ± 4.095		64.549 ± 4.178[d]
Recovery		91.624 ± 6.633		102.430 ± 8.787

a Mean ± SE of four rats per group.
b Based on 7% of body weight.
c Based on 40% of body weight.
d Significantly different ($p < 0.05$) from males.

TABLE 2

TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY 24 HR AFTER ORAL ADMINISTRATION OF ^{14}C-TNT (100 mg/kg) TO SWISS MICE[a]

Tissue/Excretum	Males		Females	
	Concentration (µg eq/g or ml)	Percent of Dose	Concentration (µg eq/g or ml)	Percent of Dose
Blood[b]	6.936 ± 4.297	0.498 ± 0.311	1.023 ± 0.130[d]	0.069 ± 0.007[d]
Liver	14.845 ± 3.594	0.756 ± 0.201	8.372 ± 1.108[d]	0.500 ± 0.073[d]
Kidneys	29.698 ± 21.768	0.541 ± 0.418	5.056 ± 0.539[d]	0.070 ± 0.005[d]
Lungs	9.504 ± 3.239	0.057 ± 0.018	6.383 ± 1.464	0.034 ± 0.009
Spleen	4.498 ± 2.004	0.007 ± 0.002	2.725 ± 0.605	0.006 ± 0.001
Brain	1.751 ± 0.595	0.026 ± 0.008	0.932 ± 0.186	0.017 ± 0.003
Muscle[c]	1.943 ± 0.775	0.794 ± 0.320	1.398 ± 0.308	0.480 ± 0.128
GI Tract plus contents	7.450 ± 2.163	13.453 ± 3.339	7.963 ± 0.604	7.421 ± 0.796[d]
Feces		22.012 ± 1.210		8.959 ± 1.059[d]
Urine		41.910 ± 6.785		42.874 ± 3.985
Recovery		80.058 ± 5.224		60.435 ± 2.599[d]

a Mean ± SE of seven male or eight female mice.
b Based on 7% body weight.
c Based on 40% of body weight.
d Significantly different ($p < 0.05$) from males.

TABLE 3

TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY 24 HR AFTER ORAL ADMINISTRATION OF ^{14}C-TNT (5 mg/kg) TO NEW ZEALAND RABBITS[a]

	Males		Females	
Tissue/Excretum	Concentration (µg eq/g or ml)	Percent of Dose	Concentration (µg eq/g or ml)	Percent of Dose
Blood[b]	0.199 ± 0.066	0.278 ± 0.099	0.277 ± 0.090	0.441 ± 0.168
Liver	1.450 ± 0.491	0.821 ± 0.327	1.681 ± 0.347	0.935 ± 0.255
Kidneys	0.549 ± 0.147	0.056 ± 0.014	0.927 ± 0.240	0.099 ± 0.031
Lungs	1.688 ± 0.665	0.146 ± 0.057	3.828 ± 2.952	0.293 ± 0.230
Spleen	0.173 ± 0.043	0.000 ± 0.000	0.296 ± 0.122	0.001 ± 0.000
Brain	0.091 ± 0.012	0.004 ± 0.000	0.127 ± 0.049	0.006 ± 0.002
Muscle[c]	0.098 ± 0.004	0.771 ± 0.072	0.195 ± 0.080	1.761 ± 0.799
GI Tract plus contents	1.493 ± 1.066	7.495 ± 5.171	1.186 ± 0.180	4.719 ± 0.704
Feces		1.776 ± 1.728		1.827 ± 0.197
Urine		66.296 ± 8.304		78.857 ± 16.304
Recovery		77.645 ± 2.683		88.940 ± 18.259

a Mean ± SE of three rabbits per group.
b Based on 7% of body weight.
c Based on 40% of body weight.

TABLE 4

TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY 24 HR AFTER ORAL ADMINISTRATION OF ^{14}C-TNT (5 mg/kg) TO BEAGLE DOGS[a]

Tissue/Excretum	Males		Females	
	Concentration (µg eq/g or ml)	Percent of Dose	Concentration (µg eq/g or ml)	Percent of Dose
Blood[b]	0.979 ± 0.142	1.383 ± 0.192	1.397 ± 0.181	1.956 ± 0.256
Liver	4.041 ± 1.017	2.176 ± 0.516	2.642 ± 0.191	1.786 ± 0.332
Kidneys	1.098 ± 0.113	0.101 ± 0.007	1.572 ± 0.154	0.162 ± 0.012
Lungs	0.705 ± 0.016	0.200 ± 0.104	1.524 ± 0.702	0.213 ± 0.094
Spleen	0.961 ± 0.064	0.183 ± 0.009	1.304 ± 0.183	0.208 ± 0.059
Brain	0.275 ± 0.032	0.038 ± 0.006	0.375 ± 0.101	0.063 ± 0.008
Muscle[c]	0.239 ± 0.010	1.940 ± 0.112	0.316 ± 0.025	2.526 ± 0.187
GI Tract plus contents	4.742 ± 3.059	9.997 ± 6.619	2.091 ± 0.941	4.396 ± 1.948
Feces		5.411 ± 3.173		16.790 ± 3.836[d]
Urine		55.918 ± 8.809		60.157 ± 1.463
Recovery		77.350 ± 5.485		88.259 ± 3.629

a Mean ± SE of three dogs per group.
b Based on 7% of body weight.
c Based on 40% of body weight.
d Significantly different ($p < 0.05$) from males.

TABLE 5

TISSUE-TO-BLOOD CONCENTRATION RATIOS IN RATS, MICE, RABBITS, AND DOGS AT 24 HR FOLLOWING ORAL ADMINISTRATION OF ^{14}C-TNT

Tissue	Rats		Mice		Rabbits		Dogs	
	Male	Female	Male	Female	Male	Female	Male	Female
Liver	3.7	3.3	3.0	8.1	7.3	6.0	4.1	1.9
Kidneys	1.2	0.6	4.3	4.9	2.8	3.3	1.0	1.1
Lungs	0.1	0.1	1.4	6.2	8.5	13.7	0.7	1.1
Spleen	0.6	1.1	0.7	2.6	0.9	1.1	1.0	0.9
Brain	0.1	0.1	0.3	0.9	0.5	0.5	0.3	0.3
Muscle	0.3	0.5	0.3	1.3	0.5	0.7	0.2	0.2
Blood (µg/ml)	1.0 (2.88)	1.0 (4.18)	1.0 (6.94)	1.0 (1.02)	1.0 (0.20)	1.0 (0.28)	1.0 (0.98)	1.0 (1.4)

TABLE 6

LEVELS OF RADIOACTIVITY IN BLOOD FOLLOWING ORAL OR DERMAL ADMINISTRATION OF ^{14}C-TNT (50 mg/kg) TO RATS[a]

Time After Treatment (hr)	Concentration (µg eq/ml)			
	Oral		Dermal	
	Males	Females	Males	Females
4	4.62 ± 0.65	5.82 ± 0.62	0.96 ± 0.13[b]	1.42 ± 0.23[b]
8	5.73 ± 0.41	7.41 ± 0.53	1.33 ± 0.16[b]	2.23 ± 0.31[b]
24	1.77 ± 0.23	2.72 ± 0.19	1.90 ± 0.18	2.43 ± 0.33

a Mean ± S.E. of three rats per treatment.
b Significantly different ($p < 0.05$) from oral treatment.

TABLE 7

TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY 24 HR AFTER ORAL OR DERMAL ADMINISTRATION OF ^{14}C-TNT (50 mg/kg) TO MALE SPRAGUE-DAWLEY RATS[a]

Tissue/Excretum	Oral		Dermal	
	Concentration (µg eq/g or ml)	Percent of Dose	Concentration (µg eq/g or ml)	Percent of Dose
Blood[b]	1.77 ± 0.22	0.25 ± 0.03	1.44 ± 0.19	0.23 ± 0.03
Liver	7.29 ± 0.48	0.45 ± 0.03	2.81 ± 0.31[e]	0.15 ± 0.01[e]
Kidneys	5.80 ± 108	0.26 ± 0.09	3.06 ± 0.42[e]	0.05 ± 0.006[e]
Lungs	2.05 ± 0.27	0.016 ± 0.002	1.44 ± 0.20	0.01 ± 0.002
Spleen	1.01 ± 0.28	0.003 ± 0.001	0.57 ± 0.16	0.002 ± 0.000
Brain	0.56 ± 0.14	0.007 ± 0.007	0.85 ± 0.14	0.011 ± 0.002
Muscle[c]	0.88 ± 0.37	0.70 ± 0.29	0.58 ± 0.15	0.46 ± 0.012
Fat	1.13 ± 0.68	-	2.39 ± 0.25[e]	-
GI Tract plus contents	228.4 ± 6.6	20.24 ± 1.85	35 ± 1.31	3.11 ± 0.30[e]
Feces		10.72 ± 0.88		1.32 ± 0.13[e]
Urine		59.54 ± 0.95		17.35 ± 2.09[e]
Recovery[d]		92.19 ± 1.91		22.76 ± 1.85[e]

a Mean ± SE of three (oral) or six (dermal) rats.
b Based on 7% of body weight.
c Based on 40% of body weight.
d Fat and skin (including site of application) are not included in the recovery estimates.
e Significantly different ($p < 0.05$) from oral treatment.

TABLE 8

TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY 24 HR AFTER ORAL OR DERMAL ADMINISTRATION OF ^{14}C-TNT (50 mg/kg) TO FEMALE SPRAGUE-DAWLEY RATS[a]

	Oral		Dermal	
Tissue/Excretum	Concentration (µg eq/g or ml)	Percent of Dose	Concentration (µg eq/g or ml)	Percent of Dose
Blood[b]	2.27 ± 0.60	0.34 ± 0.11	2.06 ± 0.26	0.29 ± 0.03
Liver	5.53 ± 0.76	0.33 ± 0.05	3.06 ± 0.33[e]	0.17 ± 0.01[e]
Kidneys	4.45 ± 0.43	0.066 ± 0.009	4.01 ± 0.35	0.06 ± 0.005
Lungs	2.10 ± 0.20	0.016 ± 0.002	1.69 ± 0.17	0.01 ± 0.001
Spleen	0.98 ± 0.18	0.003 ± 0.001	0.53 ± 0.08	0.002 ± 0.000
Brain	0.50 ± 0.07	0.007 ± 0.001	1.18 ± 0.67	0.014 ± 0.007
Muscle[c]	0.70 ± 0.17	0.56 ± 0.14	1.12 ± 0.53	0.86 ± 0.43
Fat	0.81 ± 0.18	-	3.81 ± 0.70[e]	-
GI Tract plus contents	410.9 ± 56.4	35.29 ± 3.94	55.8 ± 3.5[e]	6.40 ± 0.58[e]
Feces		2.14 ± 0.23		2.49 ± 0.31[e]
Urine		42.54 ± 1.54		14.55 ± 2.29[e]
Recovery[d]		81.30 ± 3.29		24.85 ± 1.82[e]

a Mean ± SE of three (oral) or six (dermal) rats.
b Based on 7% of body weight.
c Based on 40% of body weight.
d Fat and skin (including site of application) are not included in the recovery estimates.
e Significantly different ($p < 0.05$) from oral treatment.

TABLE 9

TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY 24 HR AFTER ORAL OR DERMAL ADMINISTRATION OF ^{14}C-TNT (50 mg/kg) TO MALE SWISS MICE[a]

Tissue/Excretum	Oral		Dermal	
	Concentration (µg eq/g or ml)	Percent of Dose	Concentration (µg eq/g or ml)	Percent of Dose
Blood[b]	0.93 ± 0.28	0.17 ± 0.03	1.23 ± 0.24	0.17 ± 0.03
Liver	4.79 ± 0.35	0.40 ± 0.03	3.32 ± 0.45[e]	0.30 ± 0.03[e]
Kidneys	3.07 ± 0.39	0.088 ± 0.01	3.08 ± 0.49	0.09 ± 0.01
Lungs	1.58 ± 0.23	0.015 ± 0.002	1.36 ± 0.20	0.014 ± 0.001
Spleen	1.08 ± 0.21	0.013 ± 0.008	0.56 ± 0.15	0.010 ± 0.006
Brain	0.42 ± 0.07	0.011 ± 0.002	0.67 ± 0.19	0.017 ± 0.004
Muscle[c]	0.49 ± 0.10	0.385 ± 0.083	0.75 ± 0.12	0.610 ± 0.088
Fat	0.71 ± 0.15	-	3.25 ± 1.51[e]	-
GI Tract plus contents	50.31 ± 3.84	10.19 ± 0.75	17.03 ± 1.18[e]	3.61 ± 0.25[e]
Feces		24.07 ± 0.83		14.17 ± 1.31[e]
Urine		59.05 ± 5.32		22.68 ± 2.44[e]
Recovery[d]		94.39 ± 2.16		41.69 ± 2.53[e]

a Mean ± SE of eight (oral) or six (dermal) mice.
b Based on 7% of body weight.
c Based on 40% of body weight.
d Fat and skin (including site of application) are not included in the recovery estimates.
e Significantly different from oral treatment ($p < 0.05$).

TABLE 10

TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY 24 HR AFTER ORAL OR DERMAL ADMINISTRATION OF ^{14}C-TNT (5 mg/kg) TO MALE NEW ZEALAND RABBITS[a]

	Oral		Dermal	
Tissue/Excretum	Concentration (µg eq/g or ml)	Percent of Dose	Concentration (µg eq/g or ml)	Percent of Dose
Blood[b]	0.260 ± 0.060	0.402 ± 0.080	0.182 ± 0.018[e]	0.256 ± 0.030[e]
Liver	0.822 ± 0.066	0.727 ± 0.040	1.041 ± 0.116	0.611 ± 0.050
Kidneys	0.350 ± 0.007	0.045 ± 0.010	0.611 ± 0.020[e]	0.072 ± 0.010[e]
Lungs	0.302 ± 0.004	0.025 ± 0.000	0.647 ± 0.119[e]	0.037 ± 0.000[e]
Spleen	0.105 ± 0.004	0.001 ± 0.000	0.147 ± 0.024	0.001 ± 0.000
Brain	0.035 ± 0.004	0.002 ± 0.000	0.085 ± 0.028[e]	0.004 ± 0.000
Muscle[c]	0.130 ± 0.059	1.110 ± 0.390	0.107 ± 0.039	0.860 ± 0.310
Fat	0.100 ± 0.009	-	0.212 ± 0.049[e]	-
GI Tract plus contents	5.562 ± 1.926	19.74 ± 7.350	2.682 ± 0.539[e]	5.758 ± 0.760[e]
Residual Bile	15.88 ± 14.72	-	2.920 ± 0.045[e]	-
Feces		5.447 ± 0.560		7.803 ± 1.200
Urine		68.07 ± 13.94		52.85 ± 1.720
Recovery[d]		95.57 ± 1.517		68.26 ± 1.105[e]

a Mean ± SE of three (oral) or four (dermal) rabbits.
b Based on 7% of body weight.
c Based on 40% of body weight.
d Fat, residual bile and skin (including site of application) are not included in the recovery estimates.
e Significantly different ($p < 0.05$) from oral treatment.

TABLE 11

TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY 24 HR AFTER ORAL OR DERMAL ADMINISTRATION OF ^{14}C-TNT (50 mg/kg) TO MALE NEW ZEALAND RABBITS[a]

	Oral		Dermal	
Tissue/Excretum	Concentration (µg eq/g or ml)	Percent of Dose	Concentration (µg eq/g or ml)	Percent of Dose
Blood[b]	2.26 (1.82, 2.70)	0.34 (0.30, 0.38)	2.19 (2.40, 1.97)	0.33 (0.34, 0.32)
Liver	8.67 (6.39, 10.95)	0.585 (0.48, 0.69)	7.33 (8.40, 6.26)	0.68 (0.75, 0.61)
Kidneys	3.73 (2.96, 4.50)	0.045 (0.04, 0.05)	6.88 (5.00, 8.75)	0.08 (0.05, 0.11)
Lungs	2.44 (2.12, 2.75)	0.025 (0.02, 0.03)	4.25 (5.60, 2.89)	0.08 (0.04, 0.12)
Spleen	1.21 (1.12, 1.30)	0.002 (0.001, 0.002)	0.96 (1.10, 0.82)	0.001 (0.001, 0.001)
Brain	0.47 (0.13, 0.80)	0.003 (0.001, 0.005)	0.47 (0.60, 0.33)	0.003 (0.003, 0.002)
Muscle[c]	0.66 (0.42, 0.90)	0.565 (0.39, 0.74)	0.62 (0.60, 0.64)	0.54 (0.48, 0.59)
Fat	1.78 (2.26, 1.30)	-	2.76 (1.80, 3.72)	-
GI Tract plus contents	76.78 (31.5 122.05)	22.66 (11.95, 33.36)	18.99 (14.50, 23.47)	5.83 (4.34, 7.32)
Residual Bile	16.67 (5.34, 28.00)	-	41.03 (26.30, 55.75)	-
Feces		5.08 (6.22, 3.93)		2.75 (2.37, 1.93)
Urine		74.34 (80.44, 68.23)		47.18 (52.03, 42.32)
Recovery[d]		103.63 (99.842, 107.417)		56.86 (60.404, 53.323)

a Average of two rabbits per treatment. Values from individual animals are shown in parentheses.
b Based on 7% of body weight.
c Based on 40% of body weight.
d Fat, residual bile and skin (including site of application) are not included in the recovery estimates.

TABLE 12

TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY 24 HR AFTER ORAL OR DERMAL ADMINISTRATION OF ^{14}C-TNT (5 mg/kg) TO MALE BEAGLE DOGS[a]

	Oral		Dermal	
Tissue/Excretum	Concentration (µg eq/g or ml)	Percent of Dose	Concentration (µg eq/g or ml)	Percent of Dose
Blood[b]	0.717 ± 0.215	1.110 ± 0.364	0.188 ± 0.026[e]	0.263 ± 0.036[e]
Liver	3.525 ± 0.156	2.402 ± 0.127	0.740 ± 0.036[e]	0.503 ± 0.030[e]
Kidneys	1.017 ± 0.183	0.130 ± 0.012	0.498 ± 0.031[e]	0.060 ± 0.003[e]
Lungs	0.817 ± 0.164	0.137 ± 0.027	0.668 ± 0.330	0.128 ± 0.060
Spleen	0.540 ± 0.140	0.060 ± 0.030	0.240 ± 0.036[e]	0.016 ± 0.003[e]
Brain	0.120 ± 0.020	0.020 ± 0.000	0.166 ± 0.086	0.030 ± 0.015
Muscle[c]	0.165 ± 0.045	1.405 ± 0.325	0.086 ± 0.006[e]	0.683 ± 0.068[e]
Fat	0.145 ± 0.035	-	0.553 ± 0.263[e]	-
GI Tract plus contents	6.439 ± 2.266	14.632 ± 6.498	1.293 ± 0.138[e]	1.682 ± 0.130[e]
Residual Bile	59.825 ± 6.325	-	39.996 ± 3.328[e]	-
Feces		8.995 ± 0.025		1.710 ± 0.380[e]
Urine		70.50 ± 2.955		11.730 ± 1.648[e]
Recovery[d]		99.391 ± 6.032		16.807 ± 1.244[e]

a Mean ± SE of three dogs per treatment.
b Based on 7% body weight.
c Based on 40% of body weight.
d Fat, residual bile and skin (including site of application) are not included in the recovery estimates.
e Significantly different ($p < 0.05$) from oral treatment.

TABLE 13

TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY 24 HR AFTER ORAL OR DERMAL ADMINISTRATION OF ^{14}C-TNT (50 mg/kg) TO MALE BEAGLE DOGS[a]

	Oral		Dermal	
Tissue/Excretum	Concentration (µg eq/g or ml)	Percent of Dose	Concentration (µg eq/g or ml)	Percent of Dose
Blood[b]	29.225	5.410	3.095	0.435
Liver	22.600	1.503	12.165	0.680
Kidneys	9.850	0.132	3.630	0.040
Lungs	8.665	0.145	2.360	0.035
Spleen	19.820	0.298	2.800	0.029
Brain	2.180	0.056	0.542	0.009
Muscle[c]	1.606	1.690	0.552	0.441
Fat	5.230	-	6.025	-
GI Tract plus contents	14.392	1.667	16.065	1.690
Residual Bile	3,129.000	-	545.460	-
Feces		22.235		0.770
Urine		61.030		11.806
Recovery[d]		94.166		15.935

a One dog per treatment.
b Based on 7% of body weight.
c Based on 40% body weight.
d Fat, residual bile and skin (including site of application) are not included in the recovery estimates.

TABLE 14

BILE/LIVER, LIVER/BLOOD, AND BILE/BLOOD CONCENTRATION RATIOS 24 HR AFTER ORAL OR DERMAL ADMINISTRATION OF ^{14}C-TNT TO MALE RABBITS AND DOGS[a]

Species	Route	Dose (mg/kg)	Concentration (µg eg/g or ml)			Ratio		
			Bile	Liver	Blood	Bile/Liver	Liver/Blood	Bile/Blood
Rabbit	Oral	5	16	0.82	0.26	19	3.2	61
		50	17	8.71	1.90	2	4.6	9
	Dermal	5	3	1.04	0.18	3	5.8	16
		50	41	7.20	1.10	6	6.5	37
Dog	Oral	5	60	3.53	0.72	17	4.9	83
		50	3,129	22.60	29.23	139	0.8	107
	Dermal	5	40	0.74	0.19	54	3.9	211
		50	545	12.17	3.10	45	3.9	176

a The ratios were calculated from liver concentrations which were not corrected for biliary ^{14}C content.

TABLE 15

TISSUE-TO-BLOOD CONCENTRATION RATIOS IN MALE RATS, MICE, RABBITS, AND DOGS AT 24 HR FOLLOWING ORAL OR DERMAL TREATMENT WITH ^{14}C-TNT

Tissue	Rats		Mice		Rabbits		Dogs	
	Oral	Dermal	Oral	Dermal	Oral	Dermal	Oral	Dermal
Liver	4.2	2.0	5.2	2.7	3.2	5.8	4.9	3.9
Kidneys	3.3	2.1	3.3	2.5	1.3	3.4	1.4	2.6
Lungs	1.2	1.0	1.7	1.1	1.2	3.6	1.1	3.5
Spleen	0.6	0.4	1.2	0.5	0.4	0.8	0.8	1.3
Brain	0.3	0.6	0.5	0.5	0.1	0.5	0.2	0.9
Muscle	0.5	0.4	0.5	0.6	0.5	0.6	0.2	0.5
Fat	0.6	1.7	0.8	2.6	0.4	1.2	0.2	2.9
Blood (μg/ml)	1.0 (1.77)	1.0 (1.44)	1.0 (0.93)	1.0 (1.23)	1.0 (0.26)	1.0 (0.18)	1.0 (0.72)	1.0 (0.1[illegible])

TABLE 16

TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY 4 HR AFTER ORAL OR INTRATRACHEAL ADMINISTRATION OF ^{14}C-TNT (50 mg/kg) TO MALE SPRAGUE-DAWLEY RATS[a]

Tissue/Excretum	Oral		Intratracheal	
	Concentration (µg eq/g or ml)	Percent of Dose	Concentration (µg eq/g or ml)	Percent of Dose
Blood[b]	9.59 ± 1.07	1.34 ± 0.14	15.62 ± 1.21[e]	2.24 ± 0.11[e]
Liver	12.21 ± 1.48	0.98 ± 0.10	13.50 ± 0.48	1.13 ± 0.05
Kidneys	11.74 ± 1.01	0.23 ± 0.01	17.48 ± 1.34[e]	0.37 ± 0.05[e]
Lungs	44.00 ± 7.86	0.38 ± 0.07	35.70 ± 4.18	0.28 ± 0.03
Spleen	3.35 ± 1.31	0.01 ± 0.00	3.19 ± 0.16	0.01 ± 0.00
Brain	4.35 ± 0.73	0.05 ± 0.01	6.48 ± 0.59[e]	0.08 ± 0.00[e]
Muscle[c]	2.44 ± 0.62	1.95 ± 0.49	4.92 ± 0.46[e]	3.93 ± 0.37[e]
Fat	30.80 ± 2.40	-	82.41 ± 5.64[e]	-
GI Tract, No Bile Collected	499.0 ± 41.35	73.70 ± 6.82	81.65 ± 7.21[e]	18.24 ± 1.03[e]
GI Tract, (Bile Collected)	(412.0 ± 37.82)	(68.29 ± 3.70)	(12.75 ± 1.02)[e]	(1.79 ± 0.02)[e]
Urine, No Bile Collected		14.63 ± 2.16		19.32 ± 3.21
Urine (Bile Collected)		(10.73 ± 1.52)		(17.50 ± 0.90)[e]
Bile		11.57 ± 2.61		19.75 ± 1.43[e]
Recovery, No Bile Collected[d]		93.27 ± 5.01		45.60 ± 3.78[e]
Recovery (Bile Collected)[d]		(95.53 ± 3.22)		(47.06 ± 1.31)[e]

a Mean ± SE of five (oral) or six (intratracheal) rats. Three (oral) or four (intratracheal) rats had cannulated bile ducts.
b Based on 7% of body weight.
c Based on 40% of body weight.
d Fat is not included in the recovery estimates.
e Significantly different ($p < 0.05$) from oral treatment.

TABLE 17

TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY 4 HR AFTER ORAL OR INTRATRACHEAL ADMINISTRATION OF ^{14}C-TNT (50 mg/kg) TO FEMALE SPRAGUE-DAWLEY RATS[a]

	Oral		Intratracheal	
Tissue/Excretum	Concentration (µg eq/g or ml)	Percent of Dose	Concentration (µg eq/g or ml)	Percent of Dose
Blood[b]	18.33 ± 2.26	2.78 ± 0.30	30.69 ± 2.14[e]	4.29 ± 0.32[e]
Liver	9.61 ± 1.03	0.75 ± 0.11	14.25 ± 0.78	1.13 ± 0.09
Kidneys	19.14 ± 2.41	0.36 ± 0.05	23.15 ± 1.67	0.46 ± 0.03
Lungs	21.35 ± 2.70	0.25 ± 0.04	23.58 ± 3.89	0.27 ± 0.03
Spleen	2.03 ± 0.62	0.01 ± 0.33	5.84 ± 0.48[e]	0.03 ± 0.00[e]
Brain	9.44 ± 0.79	0.13 ± 0.02	16.21 ± 1.21[e]	0.27 ± 0.03[e]
Muscle[c]	6.95 ± 0.82	4.62 ± 0.53	11.34 ± 0.79[e]	8.42 ± 0.61[e]
Fat	96.31 ± 8.21	-	154.74 ± 13.68[e]	-
GI Tract, No Bile Collected	527.3 ± 37.2	79.02 ± 5.23	39.94 ± 4.22[e]	12.06 ± 1.13[e]
GI Tract (Bile Collected)	(420.0 ± 46.2)	(64.22 ± 7.21)	(16.63 ± 1.12)[e]	(2.92 ± 0.27)[e]
Urine, No Bile Collected		10.01 ± 1.47		13.23 ± 2.01
Urine (Bile Collected)		(8.42 ± 1.13)		(12.68 ± 0.83)[e]
Bile		9.67 ± 0.74		14.51 ± 1.20[e]
Recovery, No Bile Collected[d]		97.93 ± 6.21		40.16 ± 2.03[e]
Recovery (Bile Collected)		(91.21 ± 6.37)		(44.98 ± 2.11)[e]

a Mean ± S.E. of five (oral) or six (intratracheal) rats. Three (oral) or four (intratracheal) rats had cannulated bile ducts.
b Based on 7% of body weight.
c Based on 40% of body weight.
d Fat is not included in the recovery estimates.
e Significantly different ($p < 0.05$) from oral treatment.

TABLE 18

BILE/LIVER, LIVER/BLOOD, AND BILE/BLOOD CONCENTRATION RATIOS 24 HR AFTER ORAL OR INTRATRACHEAL ADMINISTRATION OF ^{14}C-TNT (50 mg/kg) TO MALE RATS[a]

Route	Time After Dosing (hr)	Concentration (µg eg/g or ml)			Ratio		
		Bile	Liver	Blood	Bile/Liver	Liver/Blood	Bile/Blood
Oral	0.25	345		7.2			48
	0.5	567		7.5			76
	1.0	547		9.0			61
	2.0	484		9.7			50
	4.0	413	12.2	9.6	34	1.27	43
Intratracheal	0.25	1,687		35.9			47
	0.5	1,903		26.6			72
	1.0	1,460		23.6			62
	2.0	972		19.4			50
	4.0	689	13.5	15.7	51	0.86	44

a The ratios were calculated from liver concentrations which were not corrected for biliary ^{14}C content.

TABLE 19

TISSUE-TO-BLOOD CONCENTRATION RATIOS IN RATS AT 4 HR FOLLOWING ORAL OR INTRATRACHEAL ADMINISTRATION OF ^{14}C-TNT

	Males		Females	
Tissue	Oral	Intratracheal	Oral	Intratracheal
Liver	1.3	0.9	0.5	0.5
Kidneys	1.2	1.1	1.0	0.8
Lungs	4.6	2.3	1.2	0.8
Spleen	0.3	0.2	0.1	0.2
Brain	0.5	0.4	0.5	0.5
Muscle	0.3	0.3	0.4	0.4
Fat	3.2	5.3	5.3	5.0
Blood (μg/ml)	1.0 (9.59)	1.0 (15.62)	1.0 (18.33)	1.0 (30.69)

TABLE 20

ETHYL ACETATE EXTRACTABLE RADIOACTIVITY FROM URINE INCUBATED WITHOUT OR WITH β-GLUCURONIDASE

Source of Urine	Route of Administration	Percent of Total Radioactivity (A) Without β-Glucuronidase	Percent of Total Radioactivity (B) With β-Glucuronidase	Ratio B/A
Male rats	Oral	19.3	56.2	2.91
	Dermal	22.6	52.7	2.33
	Oral[a]	46.1	57.6	1.25
	Intratracheal[a]	52.3	66.9	1.28
Female rats	Oral	23.4	58.7	2.51
	Dermal	29.2	53.4	1.83
	Oral[a]	43.6	60.2	1.38
	Intratracheal[a]	38.7	64.6	1.67
Male mice	Oral	45.3	59.8	1.32
	Dermal	47.1	57.0	1.21
Male rabbit	Oral	29.0	55.1	1.90
	Dermal	36.3	55.2	1.52
Male dog	Oral	23.4	54.3	2.32
	Dermal	29.0	52.5	1.81

a Urine collected from bile duct-cannulated rats.

TABLE 21

ETHYL ACETATE EXTRACTABLE RADIOACTIVITY FROM BILE INCUBATED WITHOUT OR WITH β-GLUCURONIDASE

Source of Bile	Route of Administration	Percent of Total Radioactivity (A) Without β-Glucuronidase	Percent of Total Radioactivity (B) With β-Glucuronidase	Ratio B/A
Male Rat	Oral[a]	9.6	39.7	4.14
	Intratracheal[a]	12.2	45.3	3.71
Male Rabbit	Oral[b]	16.3	36.8	2.62
	Dermal[b]	14.1	40.9	2.90
Male Dog	Oral[b]	19.2	65.7	3.42
	Dermal[b]	22.8	71.4	3.13

a Collected from bile duct-cannulated rats.
b Residual bile.

TABLE 22

RESOLUTION OF TNT AND SOME POTENTIAL METABOLITES BY THIN-LAYER CHROMATOGRAPHY

	Compound	Solvent System and R_f Values[a]				
		I	II	V	VII	IX
1.	Trinitrotoluene (TNT)	0.706	0.624	0.612	0.742	0.638
2.	Trinitrobenzyl alcohol	0.699	0.315	0.455	0.521	0.405
3.	Trinitrobenzoic acid	0.436	0.018	0.006	0.077	0.050
4.	4-Amino-2,6-dinitrotoluene	0.661	0.339	0.376	0.497	0.380
5.	2-Amino-4,6-dinitrotoluene	0.667	0.321	0.303	0.485	0.374
6.	4,6-Diamino-2-nitrotoluene	0.536	0.089	0.112	0.166	0.123
7.	2,6-Diamino-4-nitrotoluene	0.528	0.074	0.095	0.110	0.074
8.	4-Hydroxylamino-2,6-dinitrotoluene	0.712	0.213	0.260	0.368	0.294
9.	2-Hydroxylamino-4,6-dinitrotoluene	0.687	0.343	0.308	0.490	0.393
10.	2,6,2,6-Tetranitro-4,4-azoxytoluene	0.760	0.645	0.650	0.791	0.650

a Solvent systems are:

(I) n-Butanol:acetic acid:water (10:1:1, v/v)
(II) Benzene:acetic acid (9:1, v/v)
(V) Benzene:ethylacetate (4:1, v/v)
(VII) Benzene:acetic acid (4:1, v/v)
(IX) Toluene:acetic acid (4:1, v/v)

TABLE 23

RESOLUTION OF TNT AND SOME POTENTIAL METABOLITES BY GAS CHROMATOGRAPHY

Compound	Retention Time (min)	
	Column A[a]	Columb B[b]
TNT	6.88	1.56
Trinitrobenzyl alcohol	6.3	1.6
4-Amino-2,6-dinitrotoluene	13.4	5.9
2-Amino-4,6-dinitrotoluene	16.2	7.5
4,6-Diamino-2-nitrotoluene	12.5	4.7
2,6-Diamino-4-nitrotoluene	16.3	6.6

a 10% VC-W982 on 80-100 mesh WAW-DMCS.
b 1.5% DC-LSX 30295 + 1.5% XE60 on 60-80 mesh gas chromatograph Q.

TABLE 24

RESOLUTION OF TNT AND SOME POTENTIAL METABOLITES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY[a]

Compound	Retention Time in Minutes (Relative to TNT)			
	System 1	System 2	System 3	System 4
Trinitrotoluene (TNT)	10.3 (1.0)	16.7 (1.0)	26.8 (1.0)	45.3 (1.0)
Trinitrobenzyl alcohol	17.2 (1.67)	15.5 (0.93)	11.0 (0.41)	17.0 (0.38)
2-Amino-4,6-dinitrotoluene	27.8 (2.70)	16.1 (0.96)	56.1 (2.09)	72.2 (1.59)
4-Amino-2,6-dinitrotoluene	29.9 (2.90)	16.3 (0.98)	54.4 (2.03)	71.1 (1.57)
2,6-Diamino-4-nitrotoluene	33.9 (3.29)	16.2 (0.97)	8.7 (0.33)	13.5 (0.3)
4,6-Diamino-2-nitrotoluene	40.7 (3.95)	14.0 (0.84)	11.0 (0.41)	17.0 (0.38)
2-Hydroxylamino-4,6-dinitrotoluene		16.4 (0.98)	54.4 (2.03)	71.1 (1.57)
4-Hydroxylamino-2,6-dinitrotoluene		16.8 (1.0)	60.3 (2.25)	81.2 (1.79)

a For a description of the systems used see text.

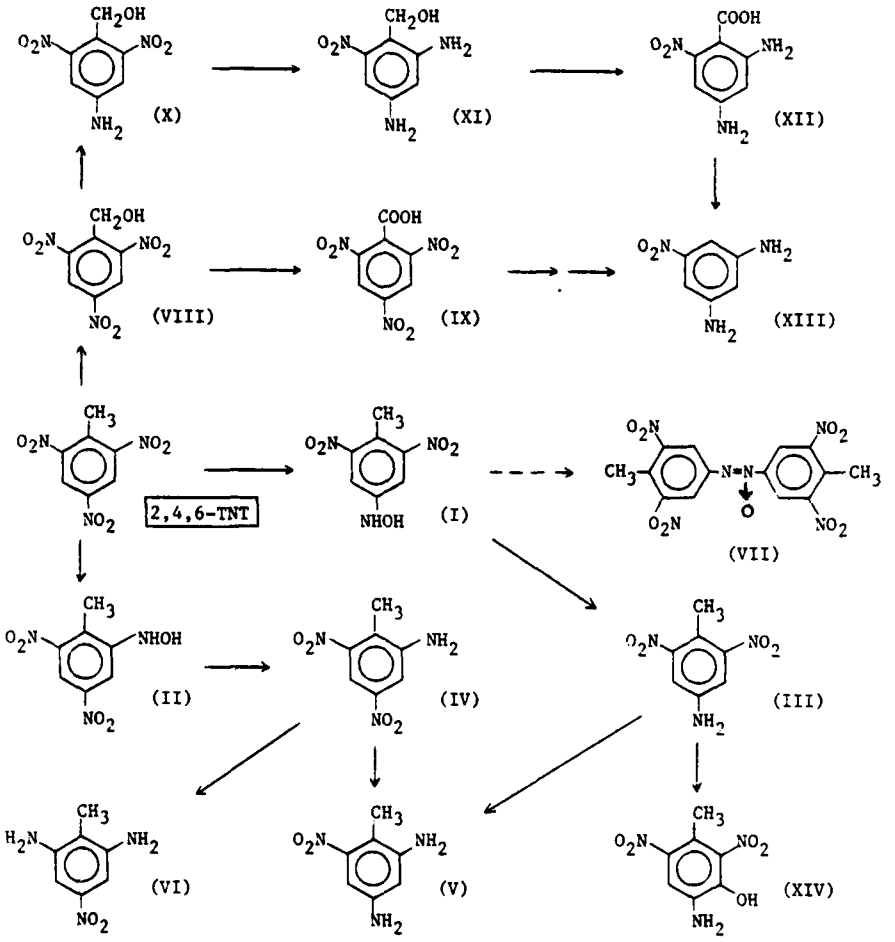

Figure 1: Schematic Presentation for Some Possible Biotransformation Products of 2,4,6-TNT

(I) 4-Hydroxylamino-2,6-dinitrotoluene
(II) 2-Hydroxylamino-4,6-dinitrotoluene
(III) 4-Amino-2,6-dinitrotoluene
(IV) 2-Amino-4,6-dinitrotoluene
(V) 4,6-Diamino-2-nitrotoluene
(VI) 2,6-Diamino-4-nitrotoluene
(VII) 2,6,2',6'-Tetranitro-4,4'-azoxytoluene
(VIII) 2,4,6-Trinitrobenzylalcohol
(IX) Trinitrobenzoic acid
(X) 4-Amino-2,6-dinitrobenzylalcohol
(XI) 2,4-Diamino-6-nitrobenzylalcohol
(XII) 2,4-Diamino-6-nitrobenzoic acid
(XIII) 5-Nitro-m-phenylenediamine
(XIV) 4-Amino-2,6-dinitro-m-cresol

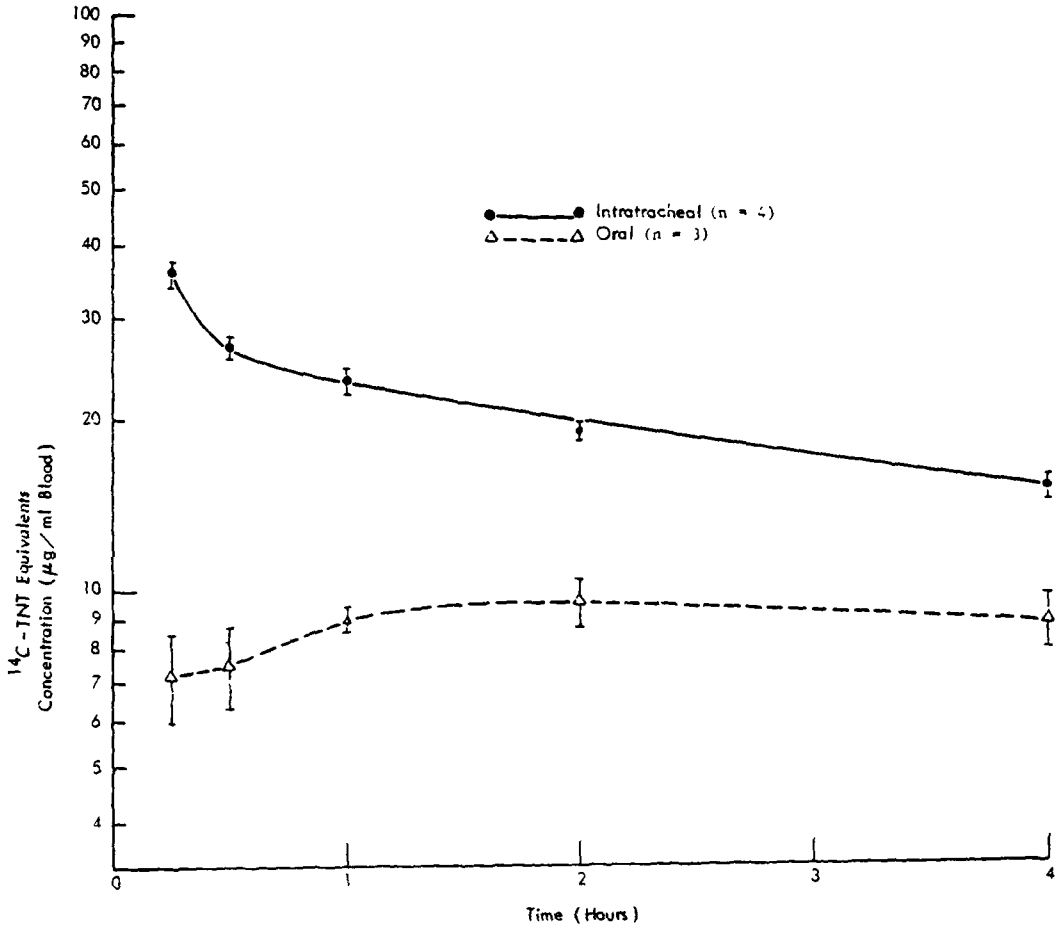

Figure 2: Levels of Radioactivity in Blood Following Oral or Intratracheal Administration of ^{14}C-TNT (50 mg/kg) to Male Sprague-Dawley Rats. Each point is the mean ± SE of 3 to 4 rats.

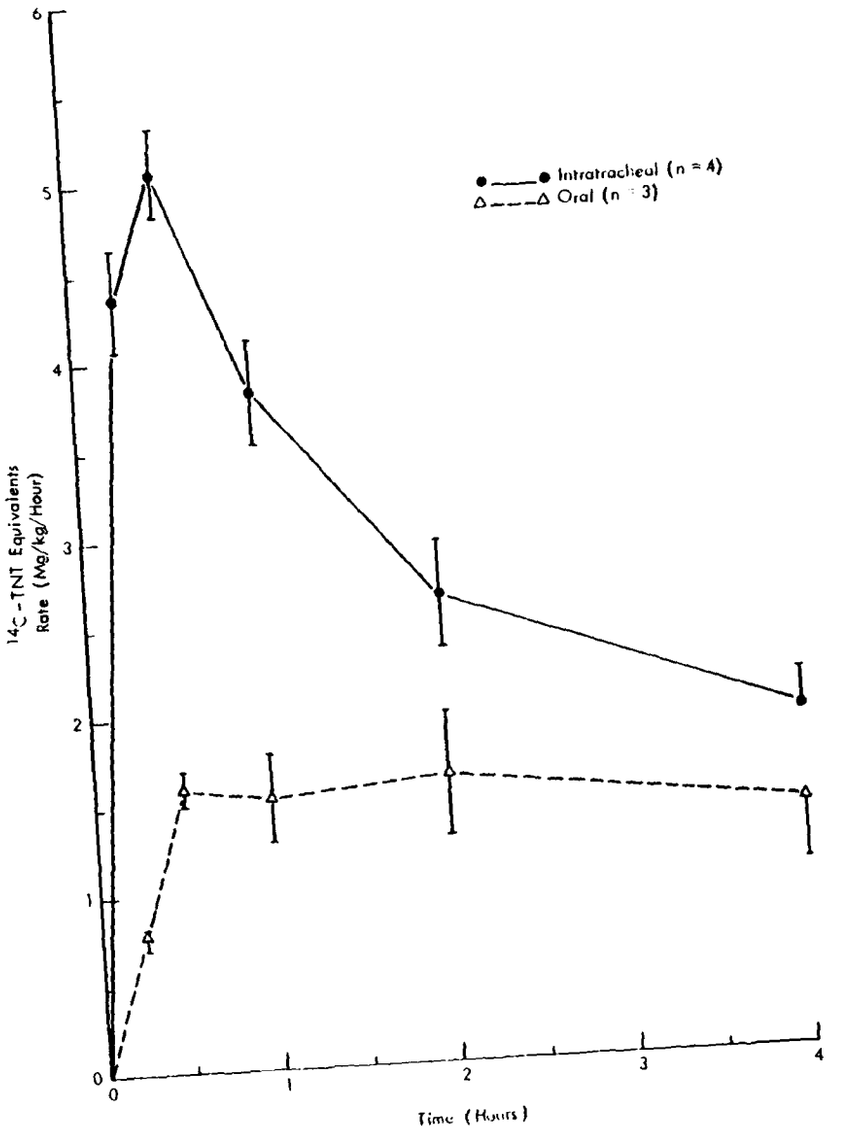

Figure 3: Rates of Excretion of Radioactivity in Bile Following Oral or Intratracheal Administration of ^{14}C-TNT (50 mg/kg) to Male Sprague-Dawley Rats. Each point is the mean ± SE of 3 to 4 rats.

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SPECIES DIFFERENCES IN THE DISPOSITION AND METABOLISM OF 2,4,6---ETC(U)
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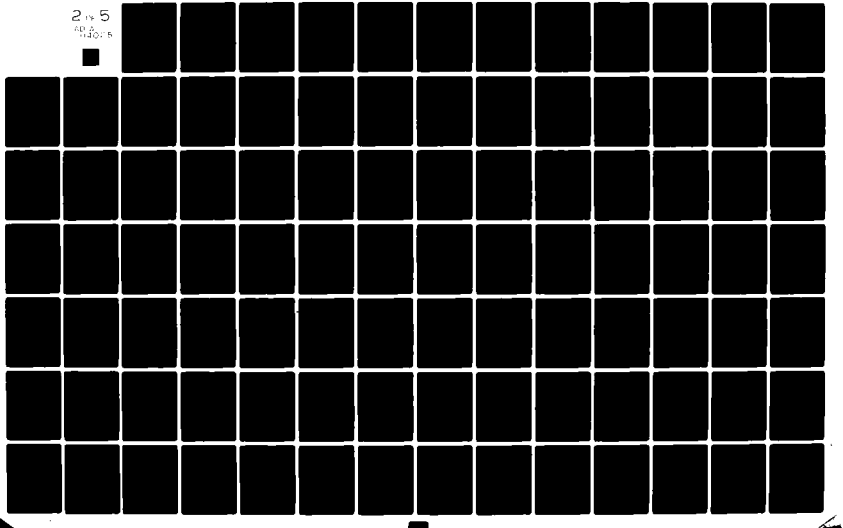

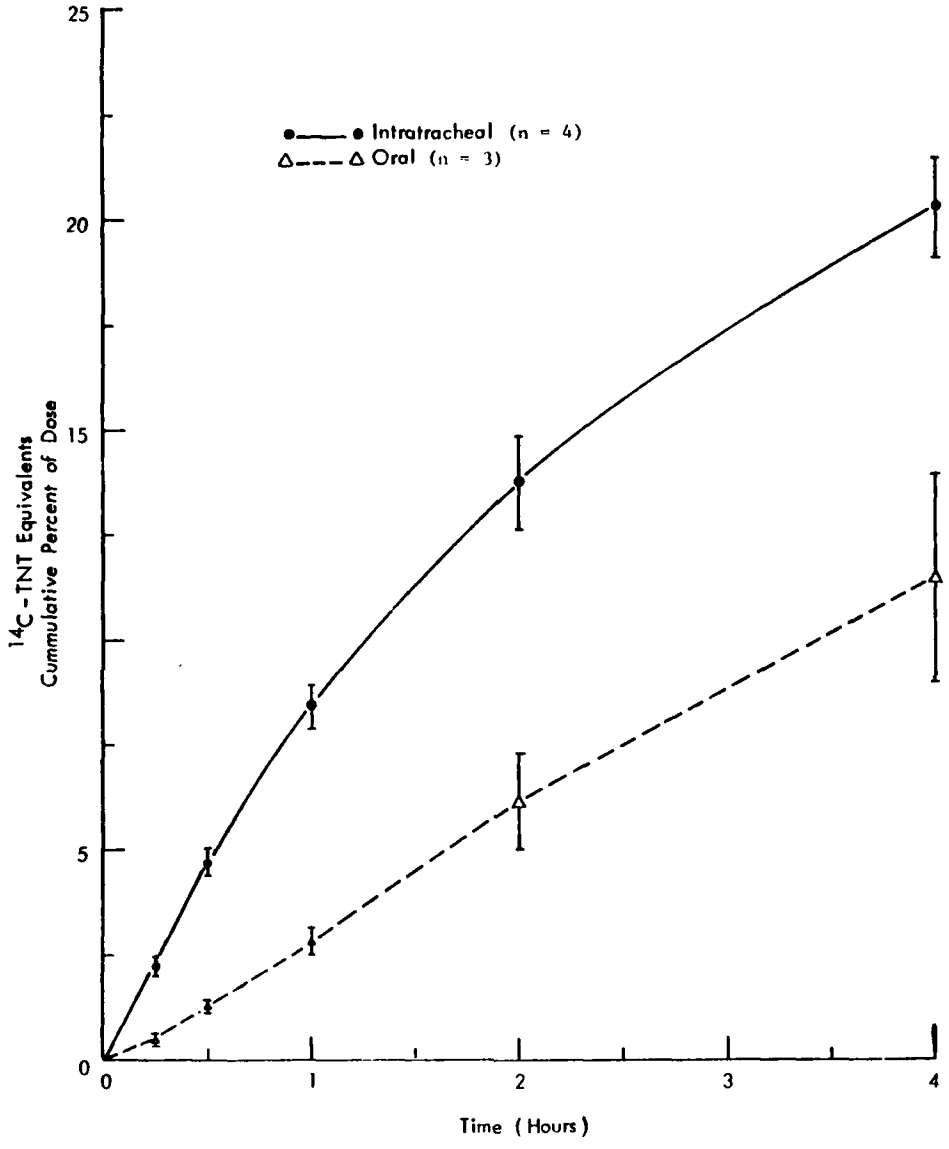

Figure 4: Cumulative Excretion of Radioactivity in Bile Following Oral or Intratracheal Administration of ^{14}C-TNT (50 mg/kg) to Male Sprague-Dawley Rats. Each point is the mean ± SE of 3 to 4 rats.

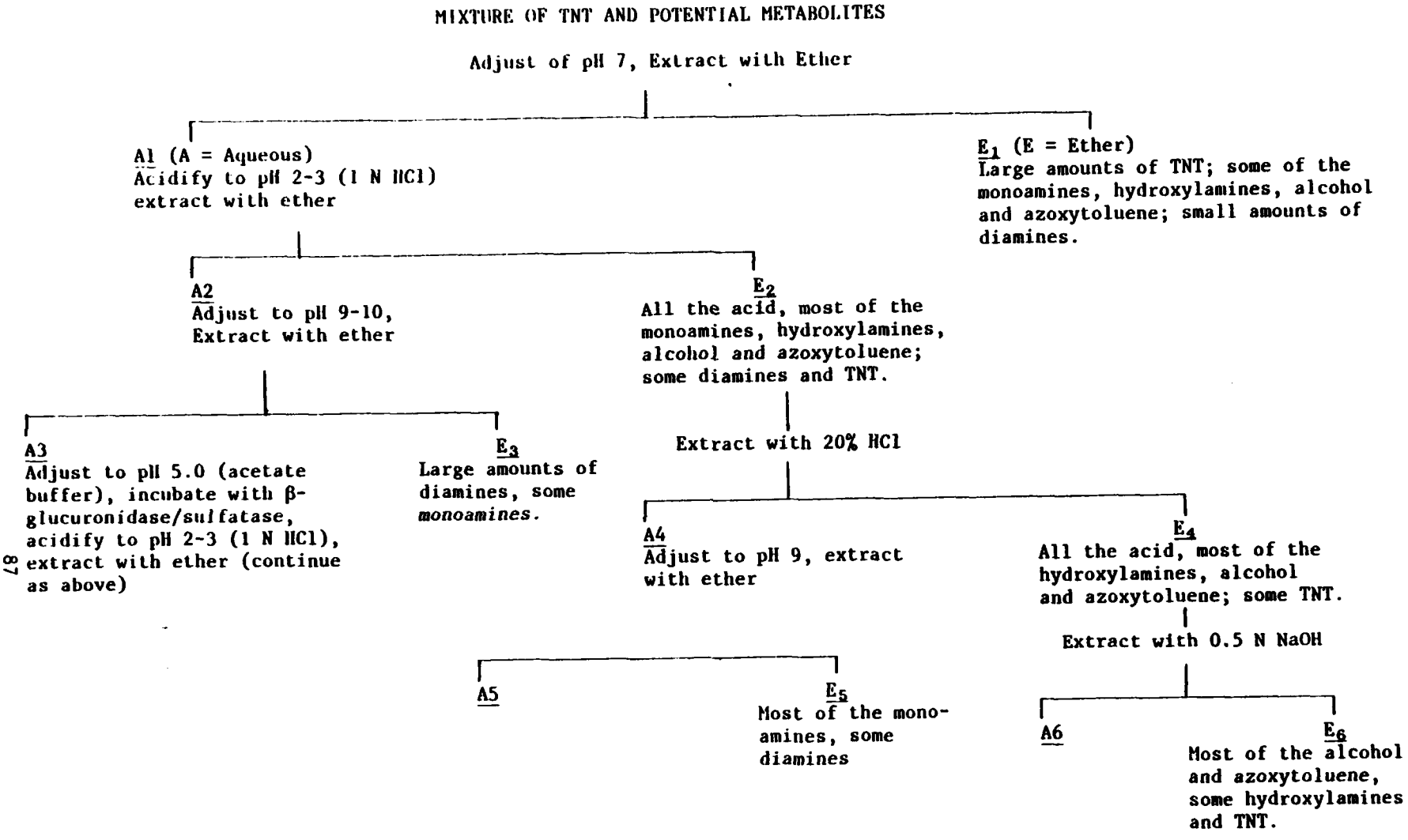

Figure 5-a: Fractionation of a Mixture of TNT and Nine Potential Metabolites by Extraction with Ether at Different pH conditions. The mixture consisted of the following:

1. TNT
2. Trinitrobenzyl alcohol
3. Trinitrobenzoic acid
4. 4-Amino-2,6-dinitrotoluene
5. 2-Amino-4,6-dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2',6'-Tetranitro-4,4'-azoxytoluene

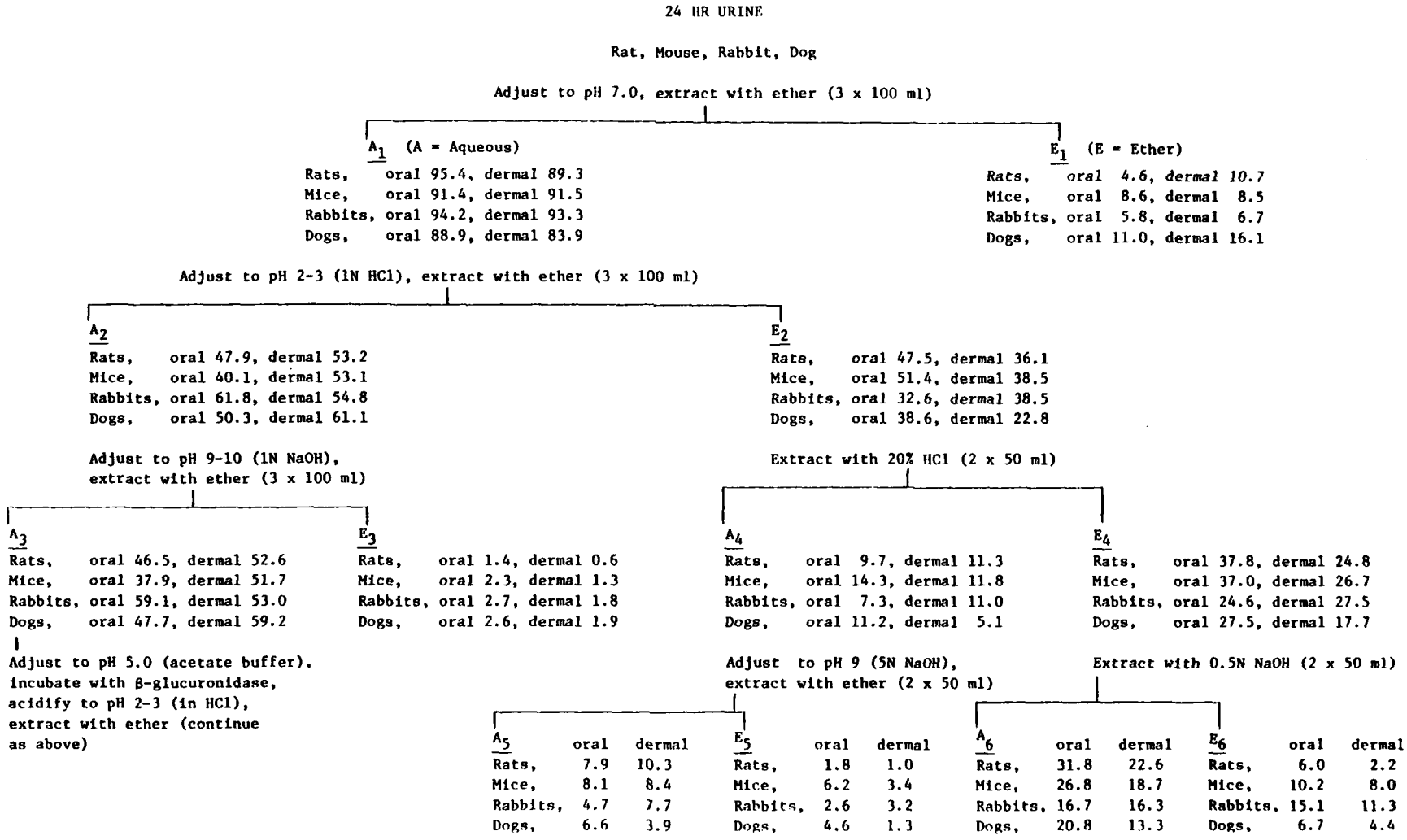

Figure 5-b: Fractionation of 24 Hr Urine Obtained from Animals Treated Orally or Dermally with ^{14}C-TNT (Values indicate the percentage of extractable radioactivity in each fraction.)

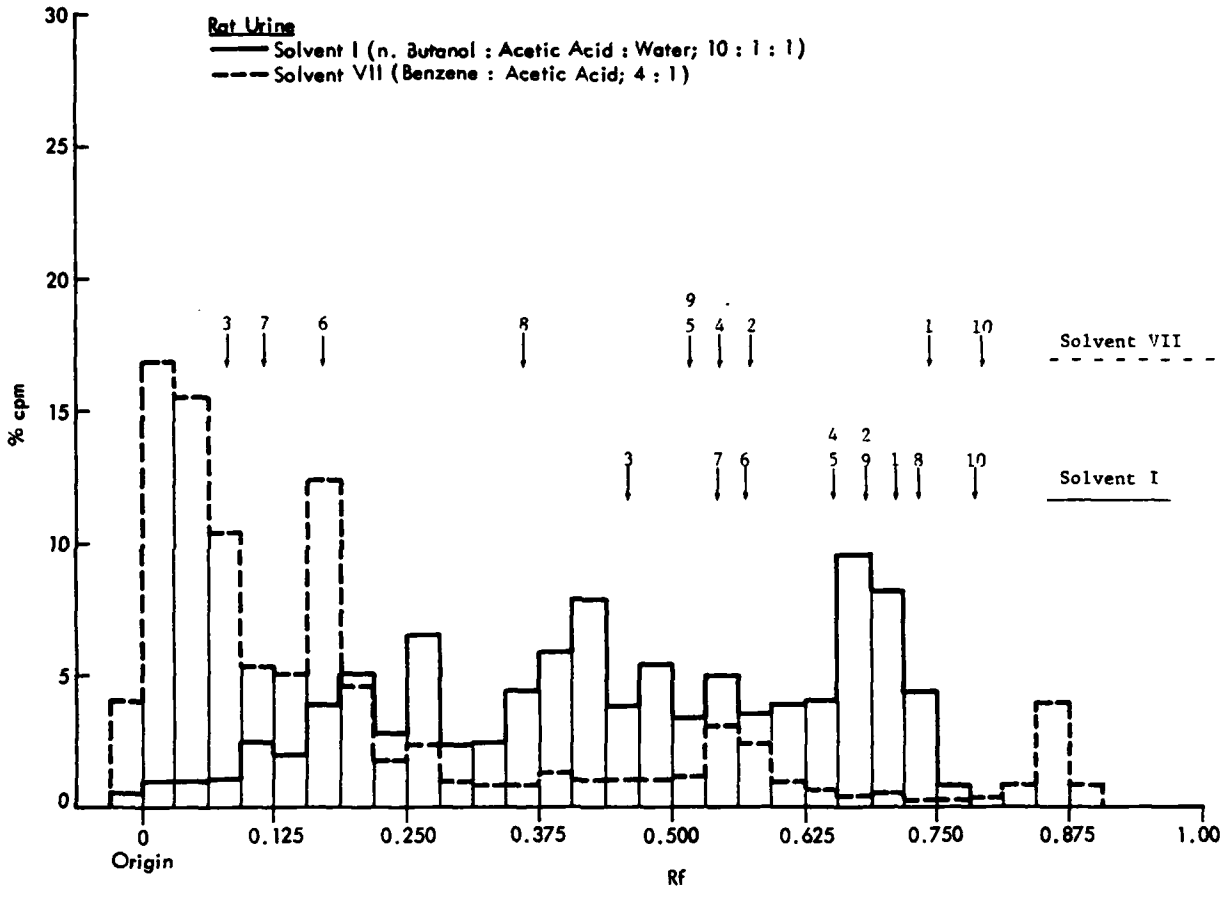

Figure 6: TLC of the Ethyl Acetate Extractable Products Obtained from Urine of Rats Treated Orally with ^{14}C-TNT (100 mg/kg). TNT and potential metabolites available as references are:

1. TNT
2. Trinitrobenzyl alcohol
3. Trinitrobenzoic acid
4. 4-Amino-2,6-dinitrotoluene
5. 2-Amino-4,6-dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2',6'-Tetranitro-4,4'-azoxytoluene

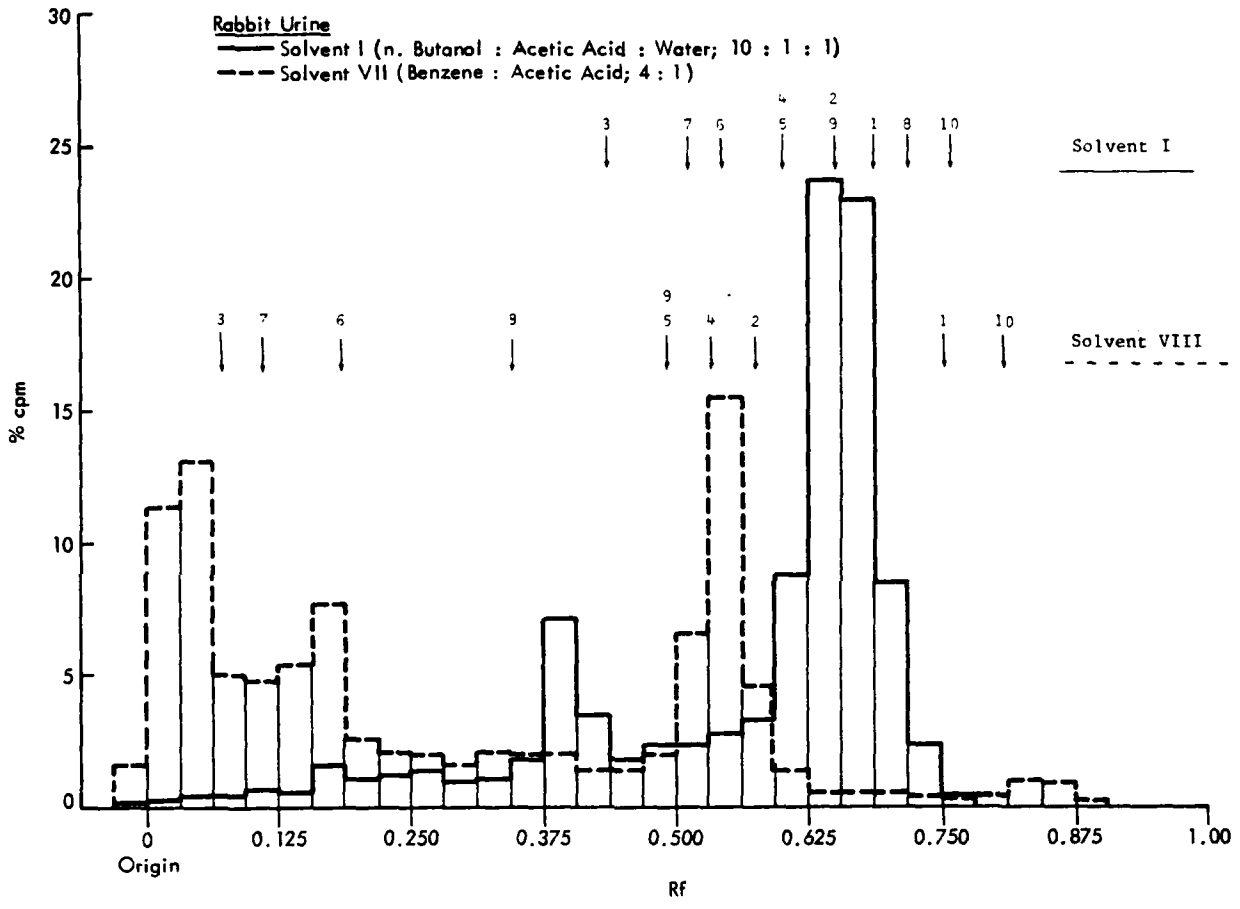

Figure 7: TLC of the Ethyl Acetate Extractable Products Obtained from *Urine of Rabbits Treated* Orally with ^{14}C-TNT (5 mg/kg). TNT and potential metabolites available as references are:

1. TNT
2. Trinitrobenzyl alcohol
3. Trinitrobenzoic acid
4. 4-Amino-2,6-dinitrotoluene
5. 2-Amino-4,6-dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2',6'-Tetranitro-4,4'-azoxytoluene

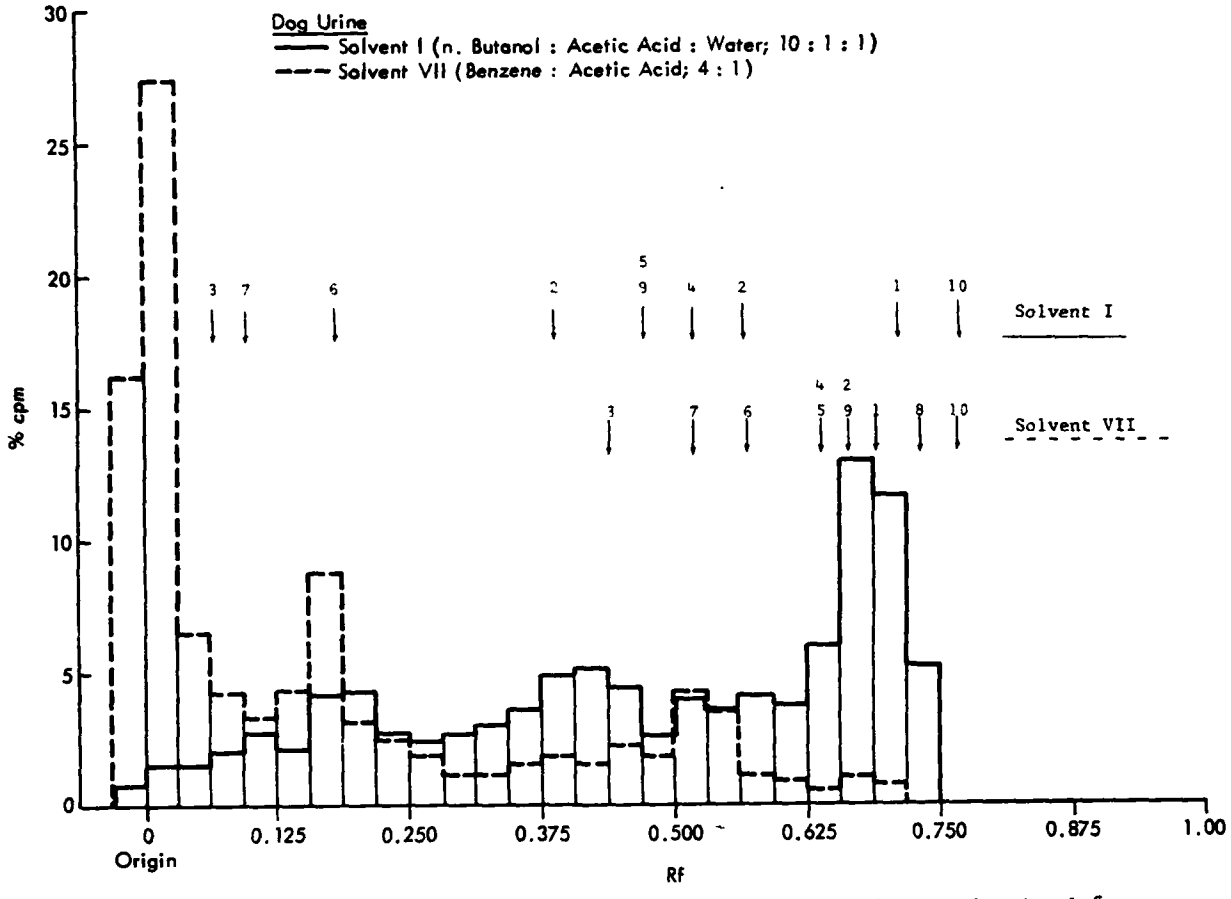

Figure 8: TLC of the Ethyl Acetate Extractable Products Obtained from Urine of Dogs Treated Orally With ^{14}C-TNT (5 mg/kg).
TNT and potential metabolites available as references are:

1. TNT
2. Trinitrobenzyl alcohol
3. Trinitrobenzoic acid
4. 4-Amino-2,6-dinitrotoluene
5. 2-Amino-4,6-dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2',6'-Tetranitro-4,4'-azoxytoluene

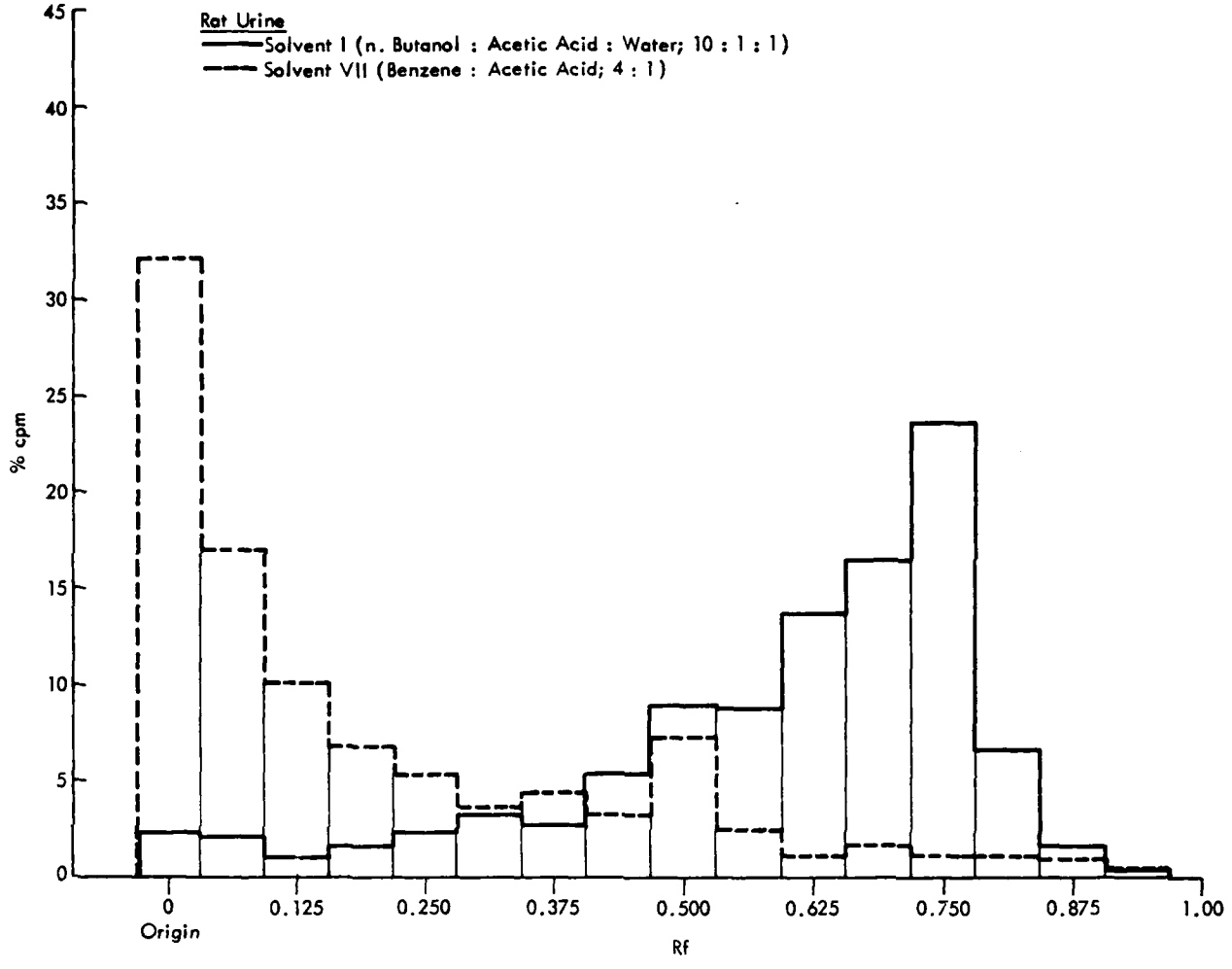

Figure 9-a: TLC of Rat Urine Obtained after Oral Administration of ^{14}C-TNT (100 mg/kg). Urine was incubated with acetate buffer (pH 5.0) for 24 hr then extracted with ethyl acetate.

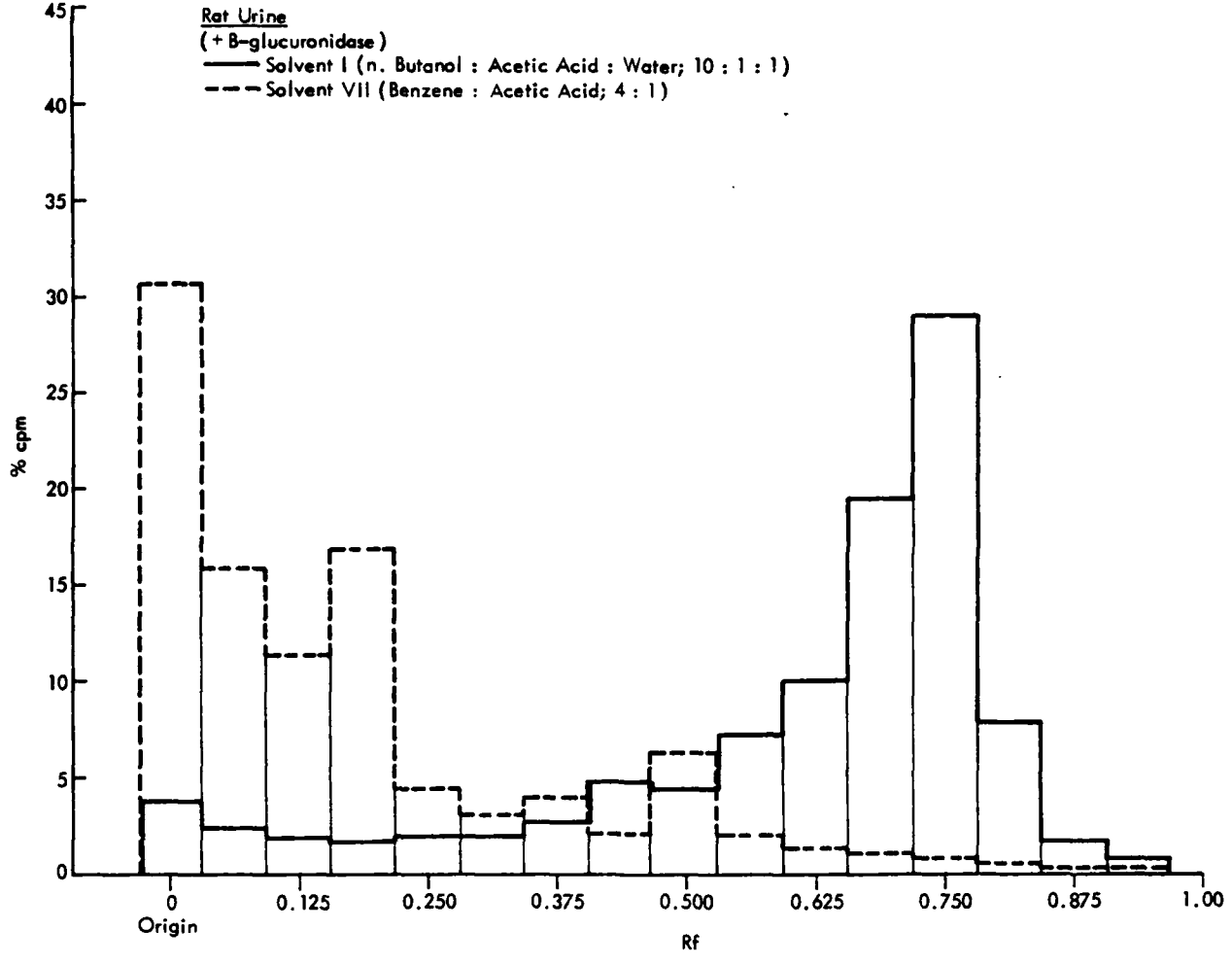

Figure 9-b: TLC of Rat Urine Obtained after Oral Administration of ^{14}C-TNT (100 mg/kg). Urine was incubated with acetate buffer (pH 5.0) and β-glucuronidase for 24 hr then extracted with ethyl acetate.

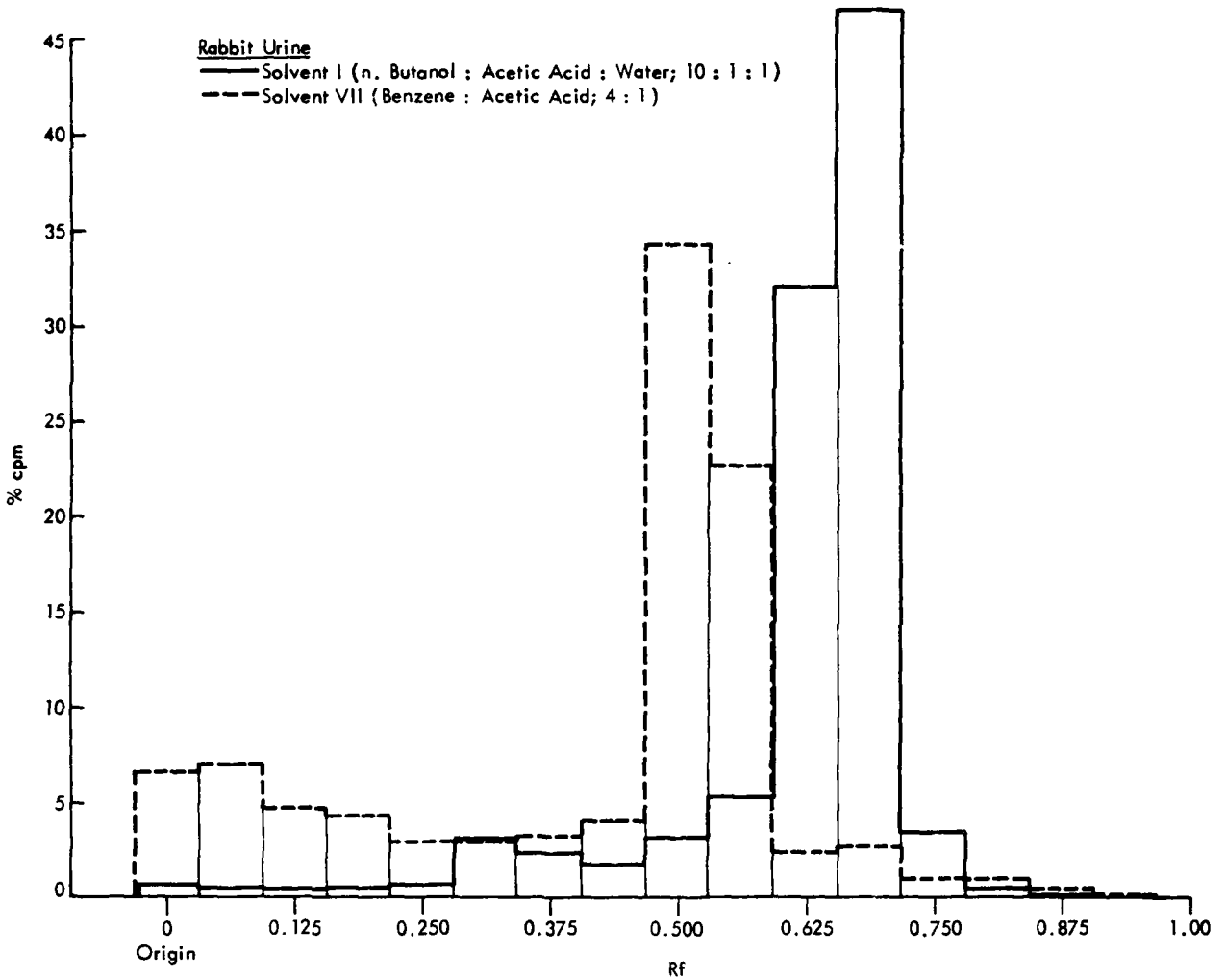

Figure 10-a: TLC of Rabbit Urine Obtained after Oral Administration of ^{14}C-TNT (5 mg/kg). Urine was incubated with acetate buffer (pH 5.0) for 24 hr then extracted with ethyl acetate.

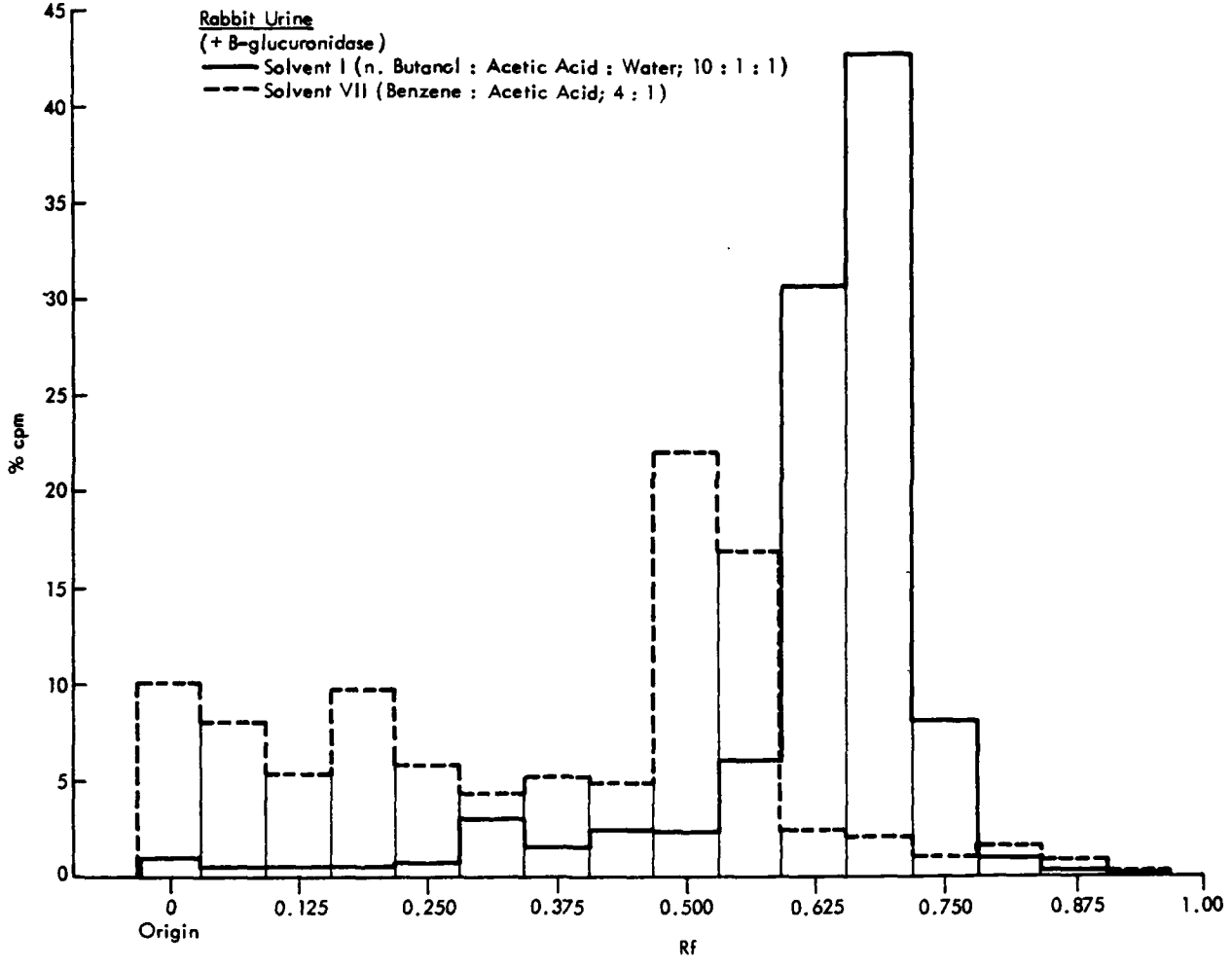

Figure 10-b: TLC of Rabbit Urine Obtained after Oral Administration of ^{14}C-TNT (5 mg/kg). Urine was incubated with acetate buffer (pH 5.0) and β-glucuronidase for 24 hr then extracted with ethyl acetate.

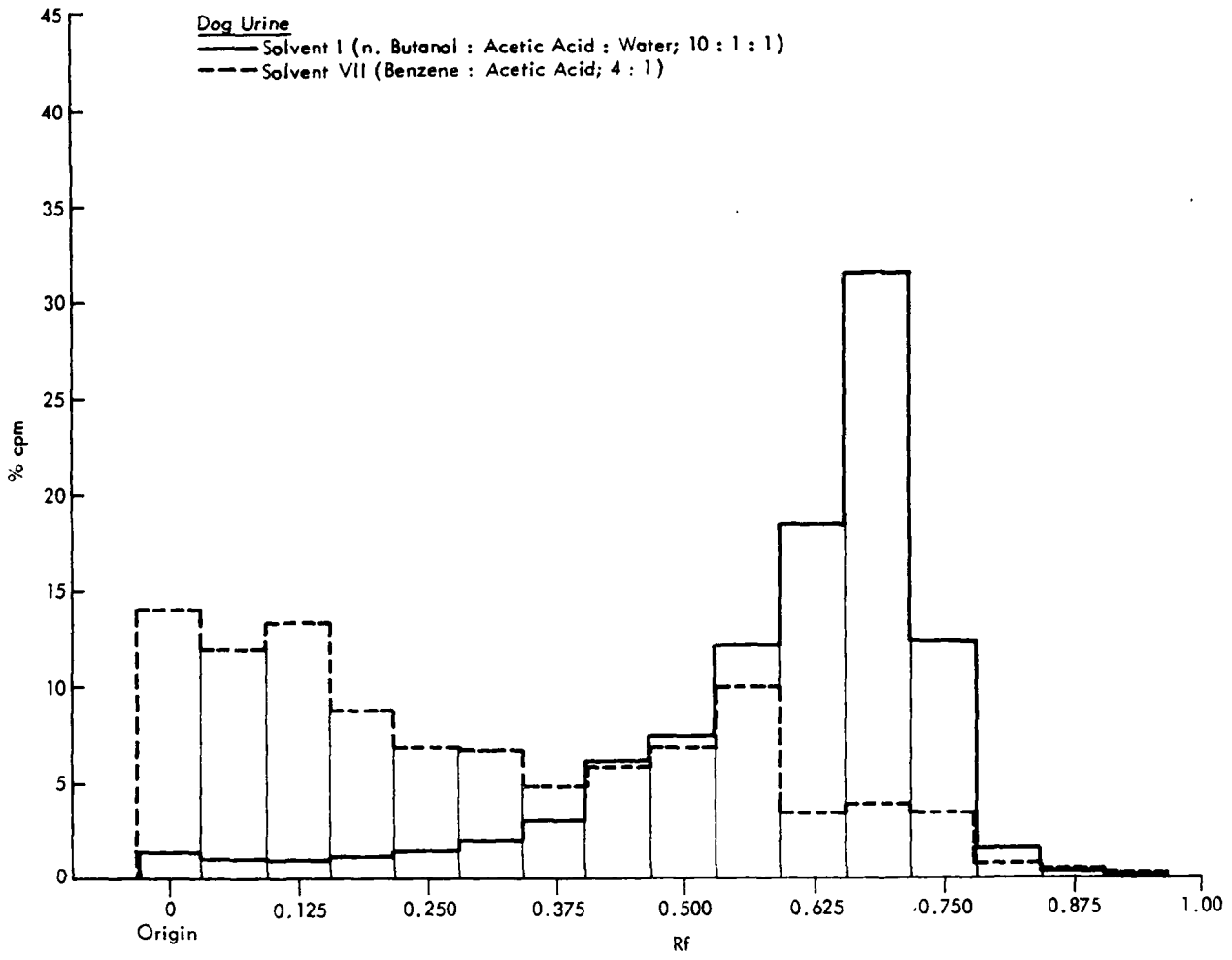

Figure 11-a: TLC of Dog Urine Obtained after Oral Administration of ^{14}C-TNT (5 mg/kg). Urine was incubated with acetate buffer (pH 5.0) for 24 hr then extracted with ethyl acetate.

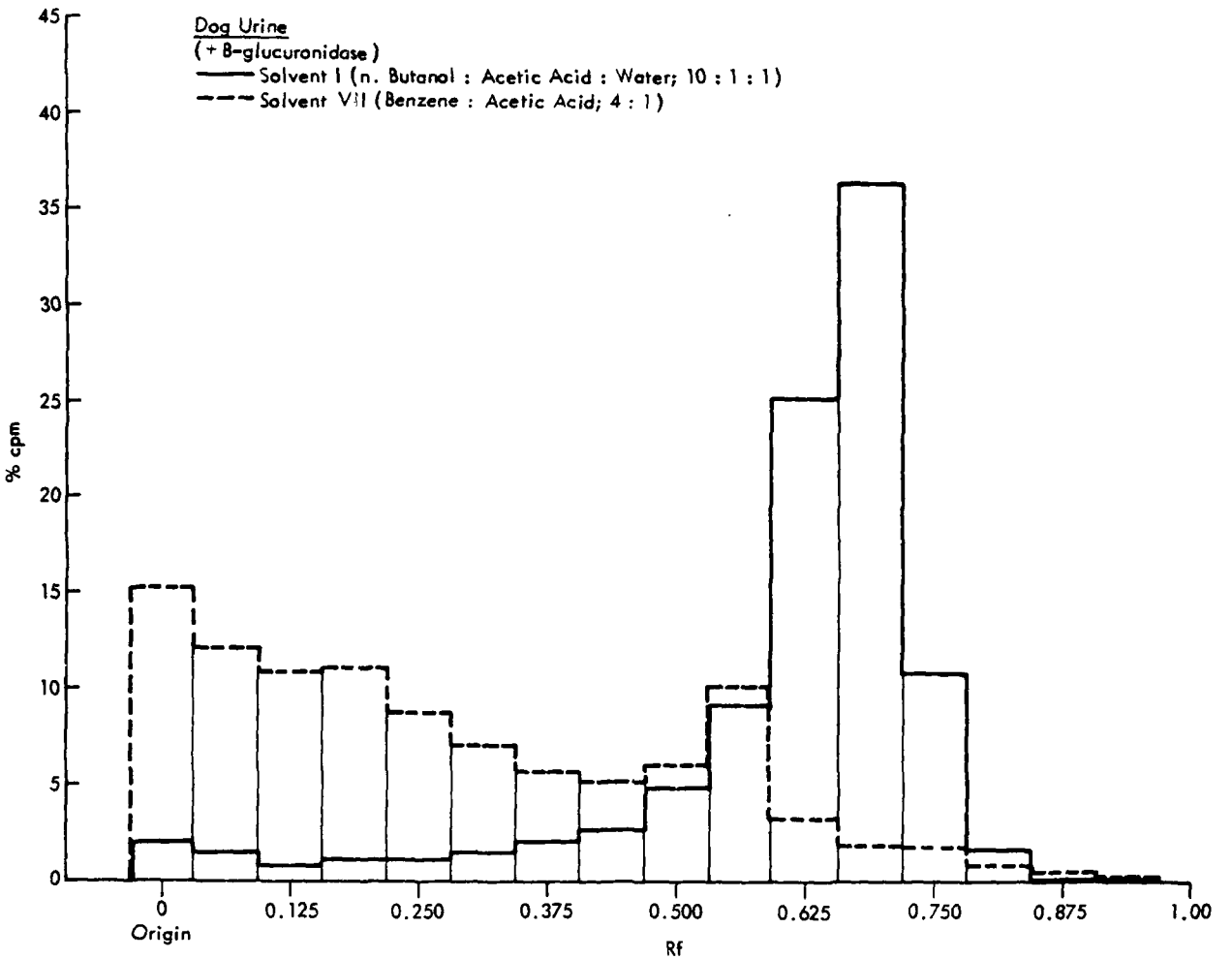

Figure 11-b: TLC of Dog Urine Obtained after Oral Administration of ^{14}C-TNT (5 mg/kg). Urine was incubated with acetate buffer (pH 5.0) and β-glucuronidase for 24 hr then extracted with ethyl acetate.

Figure 12: TLC of Raw Urine Obtained from Rats and Mice Treated Orally, Dermally or Intratracheally with ^{14}C-TNT. The TLC plates were developed in two solvent systems: I, n-butanol:acetic acid:water, 10:1:1; IX, toluene: acetic acid, 4:1. Samples of TNT and reference standards (Nos. 1-10, Table 19) were spotted and developed with the same solvents. Reference standards are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzylalcohol
3. Trinitrobenzoic Acid
4. 4-Amino-2,6-Dinitrotoluene
5. 2-Amino-4,6-Dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2,6'-Tetranitro-4,4'-azoxytoluene

Figure 12 follows

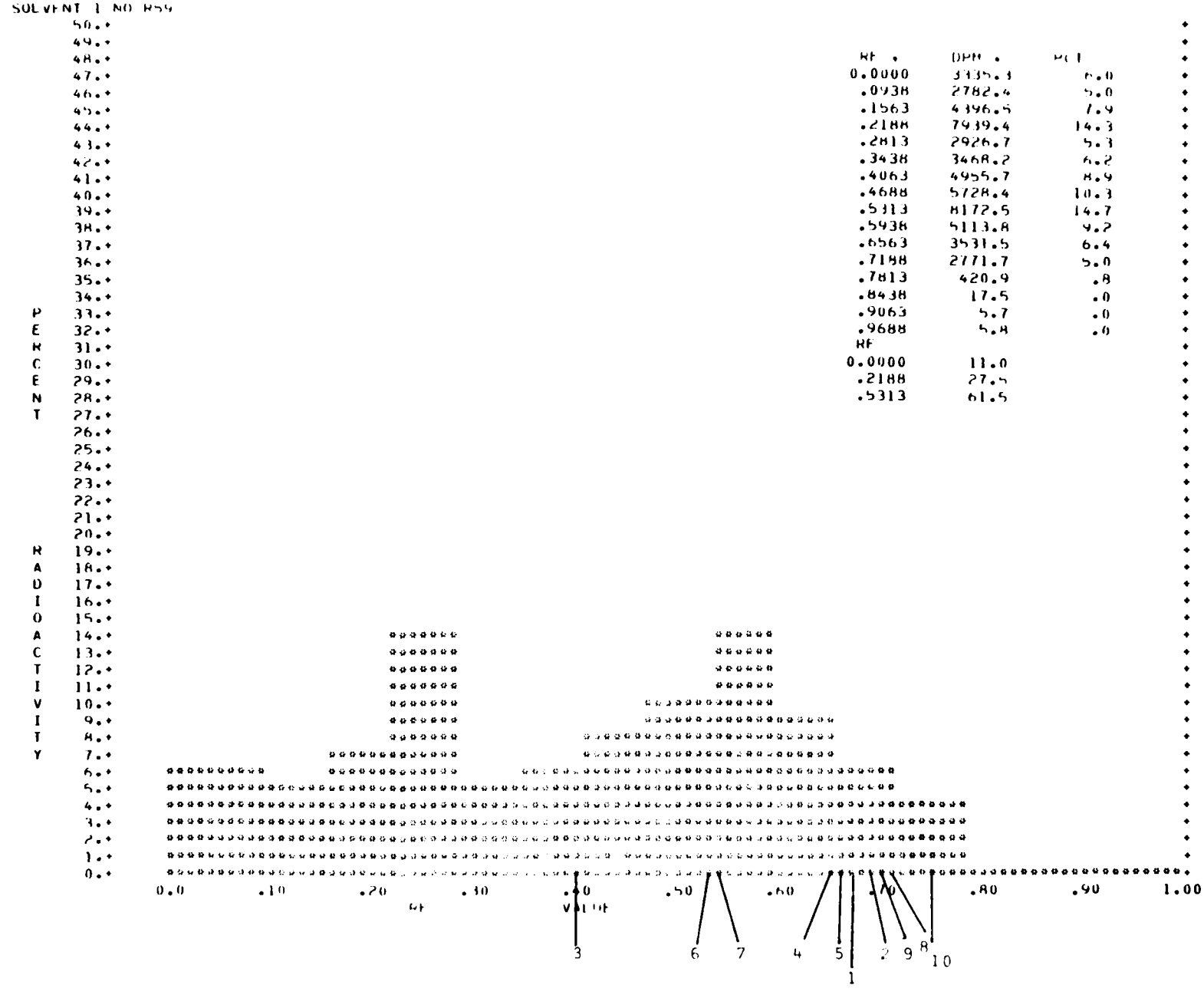

Figure 12-a-I: 24-Hr Urine, Male Rats, Oral Treatment, Solvent I

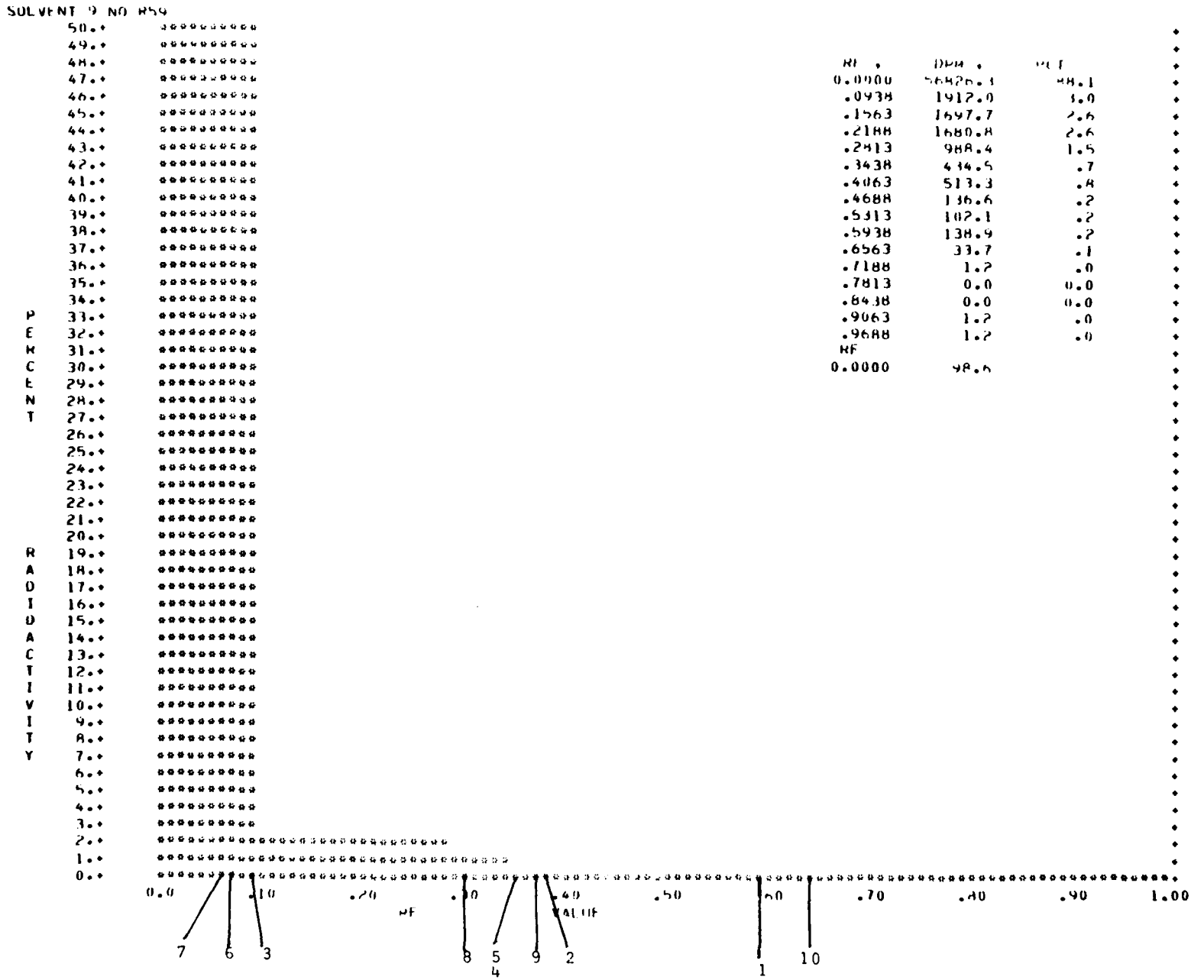

Figure 12-a-IX: 24-Hr Urine, Male Rats, Oral Treatment, Solvent IX

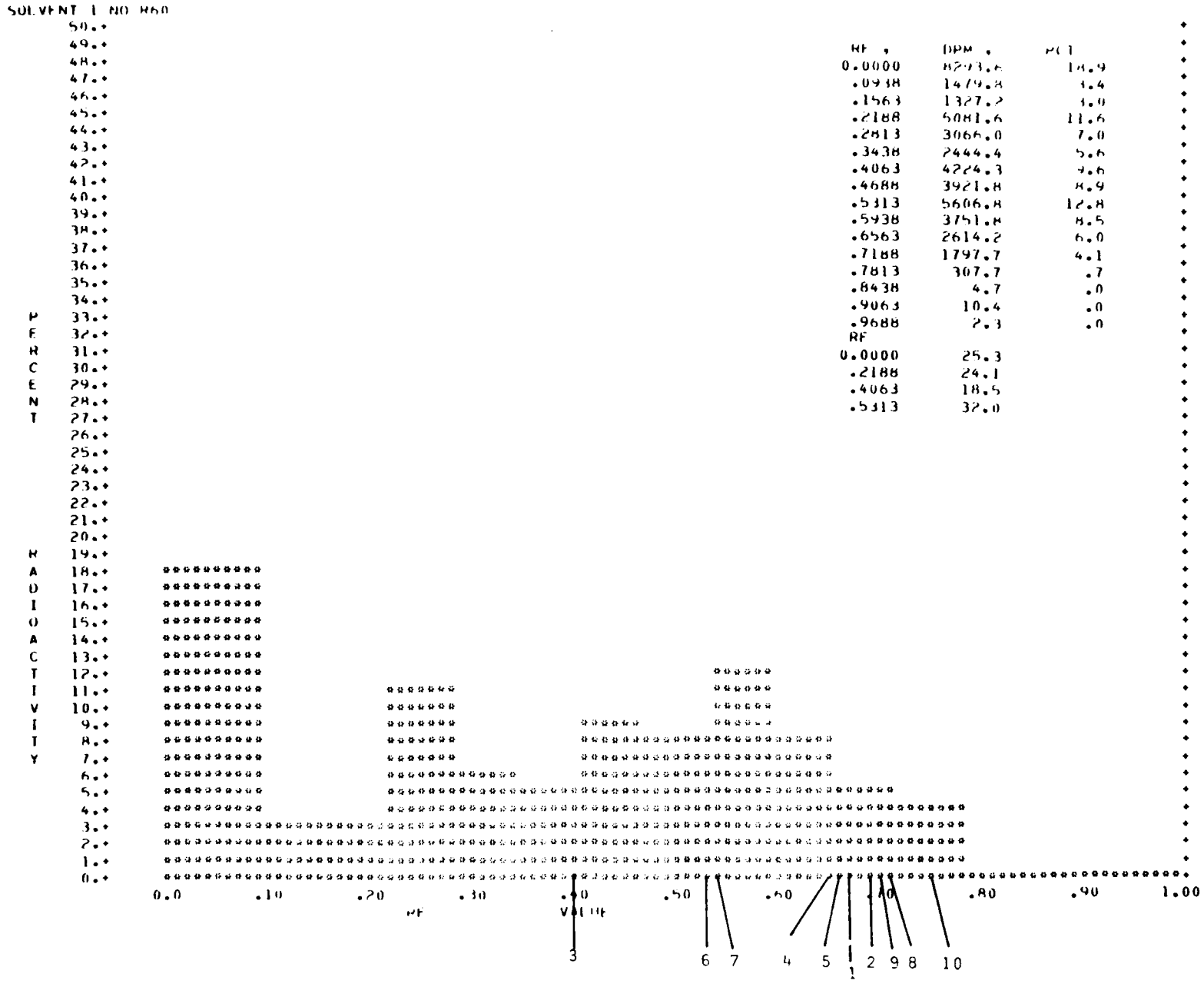

Figure 12-b-I: 24-Hr Urine, Female Rats, Oral Treatment, Solvent I

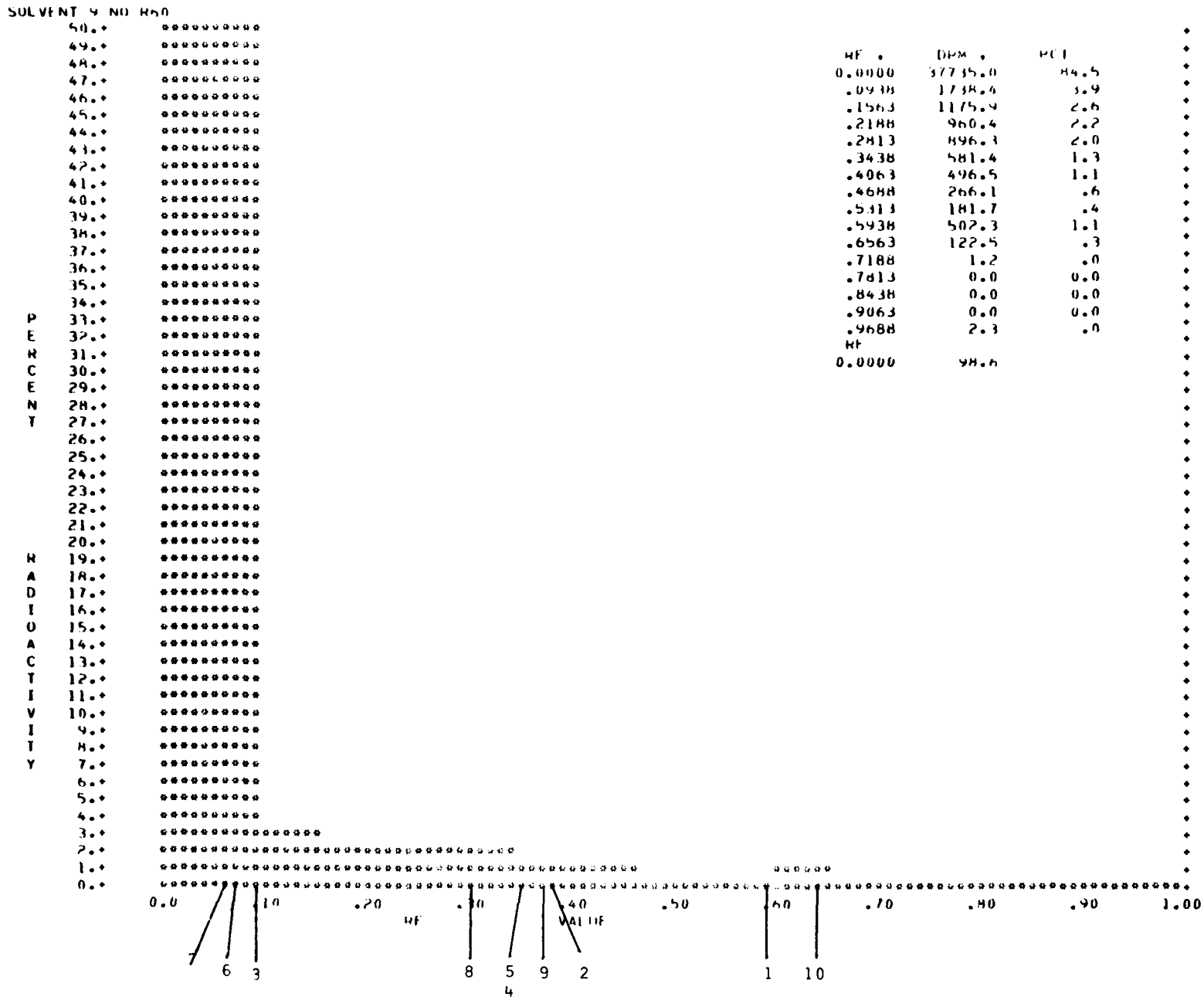

Figure 12-b-IX: 24-Hr Urine, Female Rats, Oral Treatment, Solvent IX

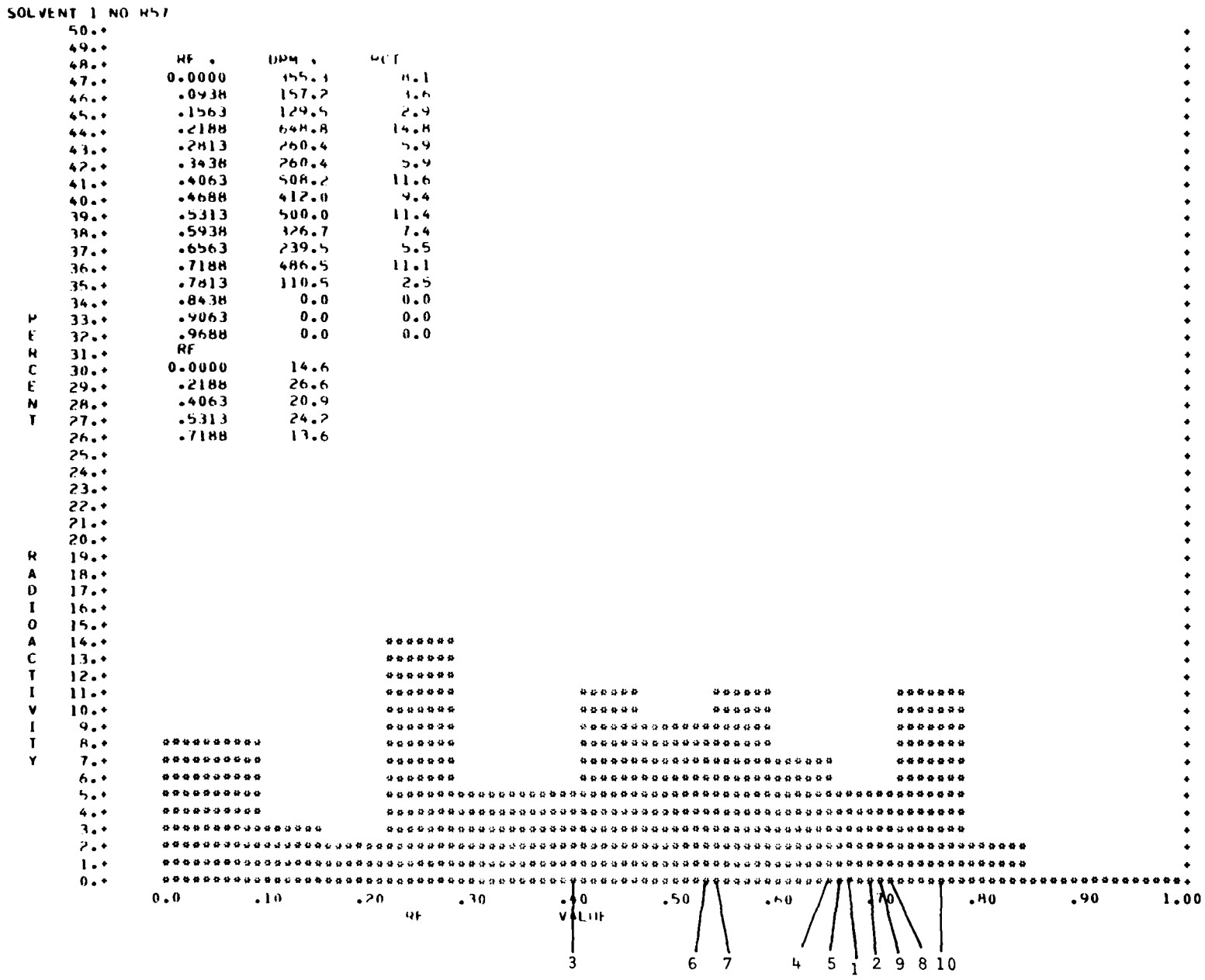

Figure 12-c-I: 24-Hr Urine, Male Rats, Dermal Application, Solvent I

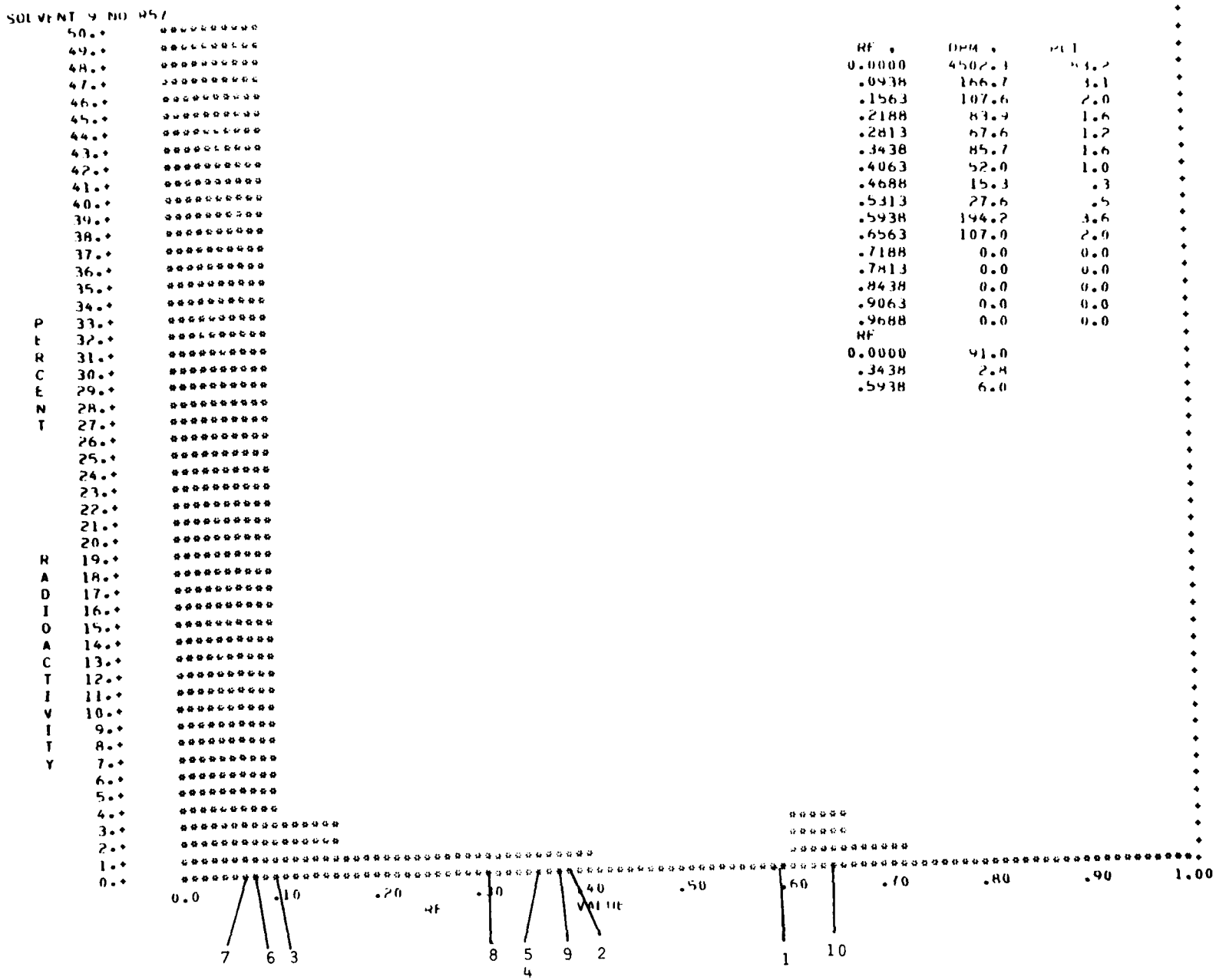

Figure 12-c-IX: 24-Hr Urine, Male Rats, Dermal Application, Solvent IX

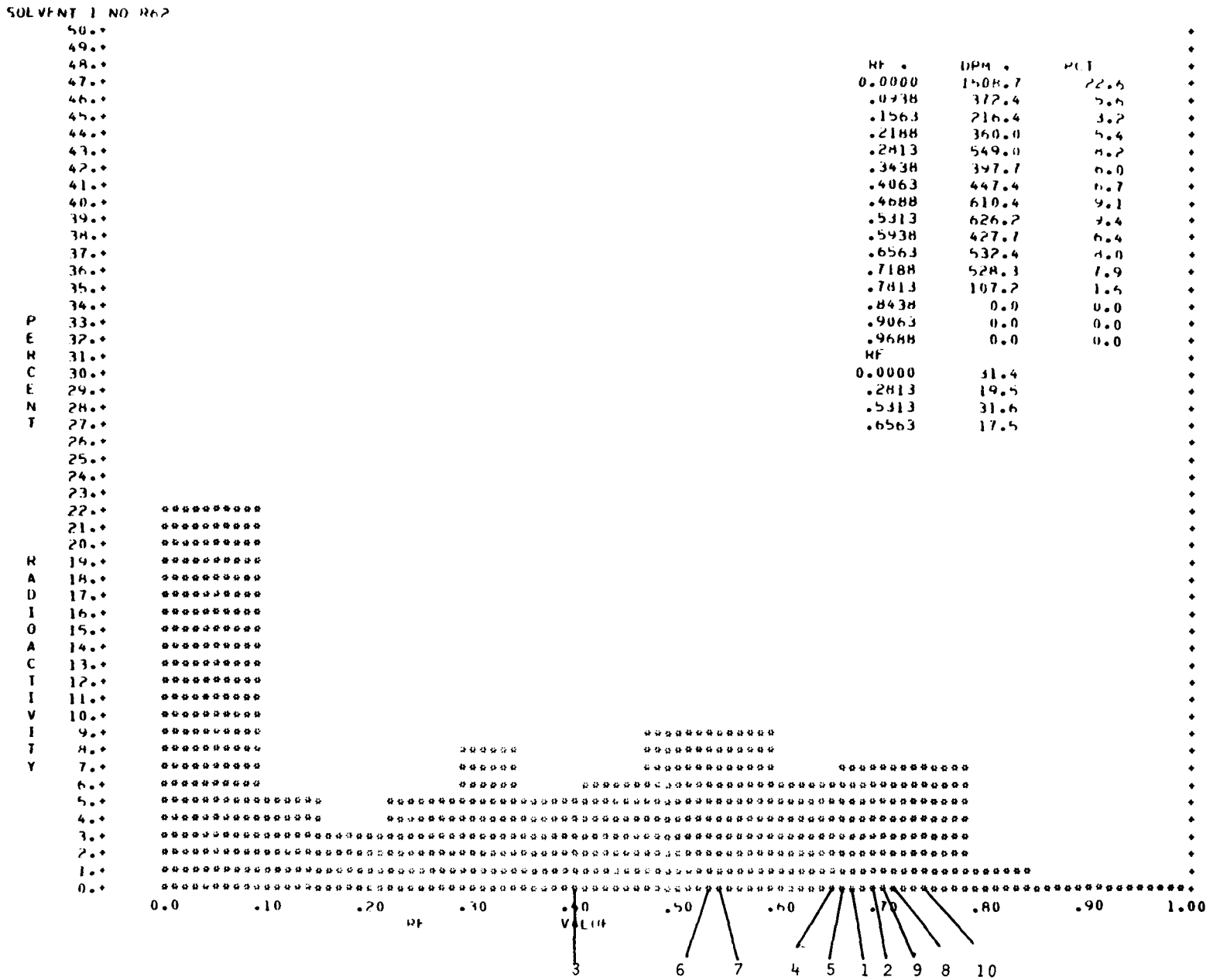

Figure 12-d-I: 24-Hr Urine, Female Rats, Dermal Application, Solvent I

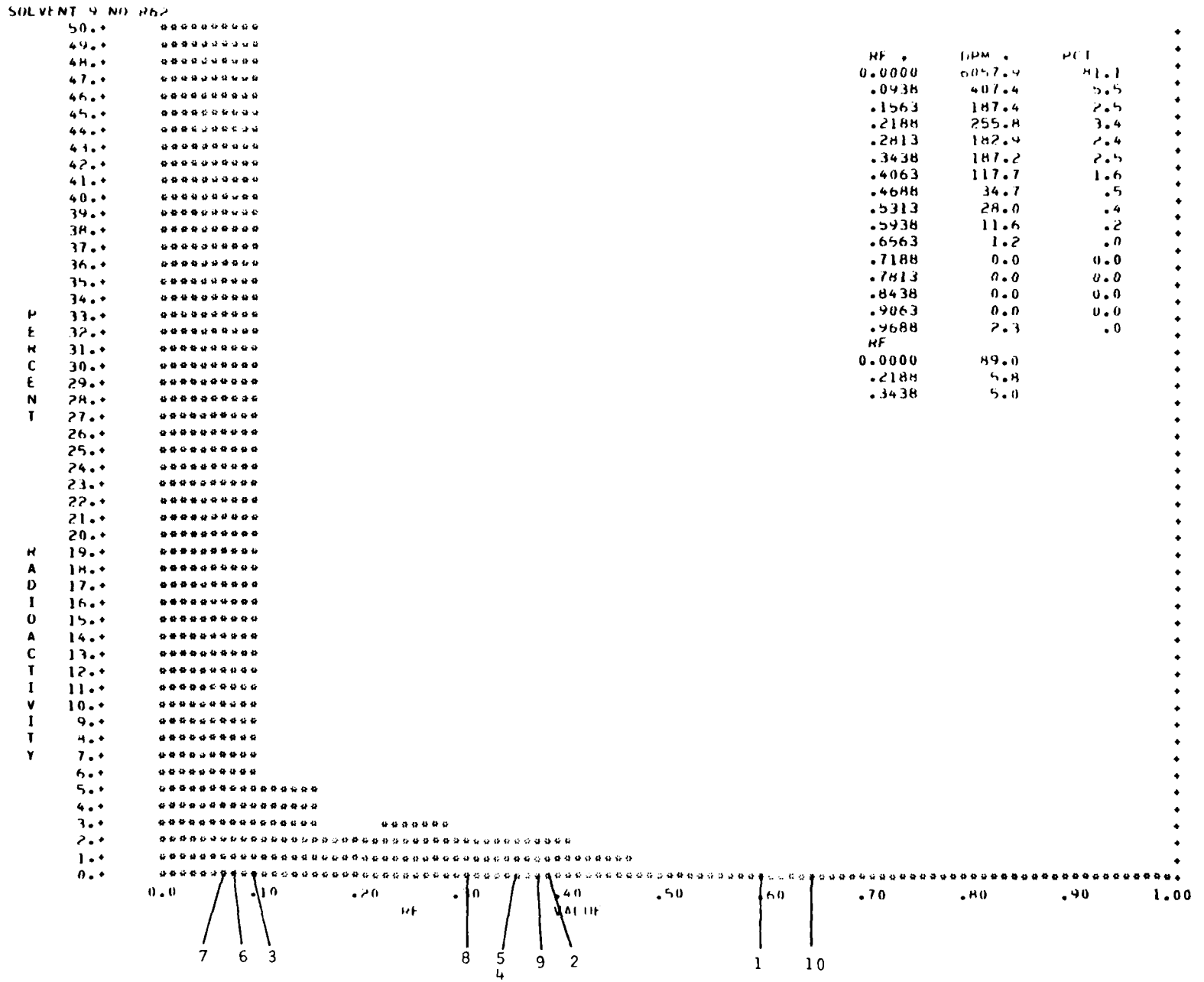

Figure 12-d-IX: 24-Hr Urine, Female Rats, Dermal Application, Solvent IX

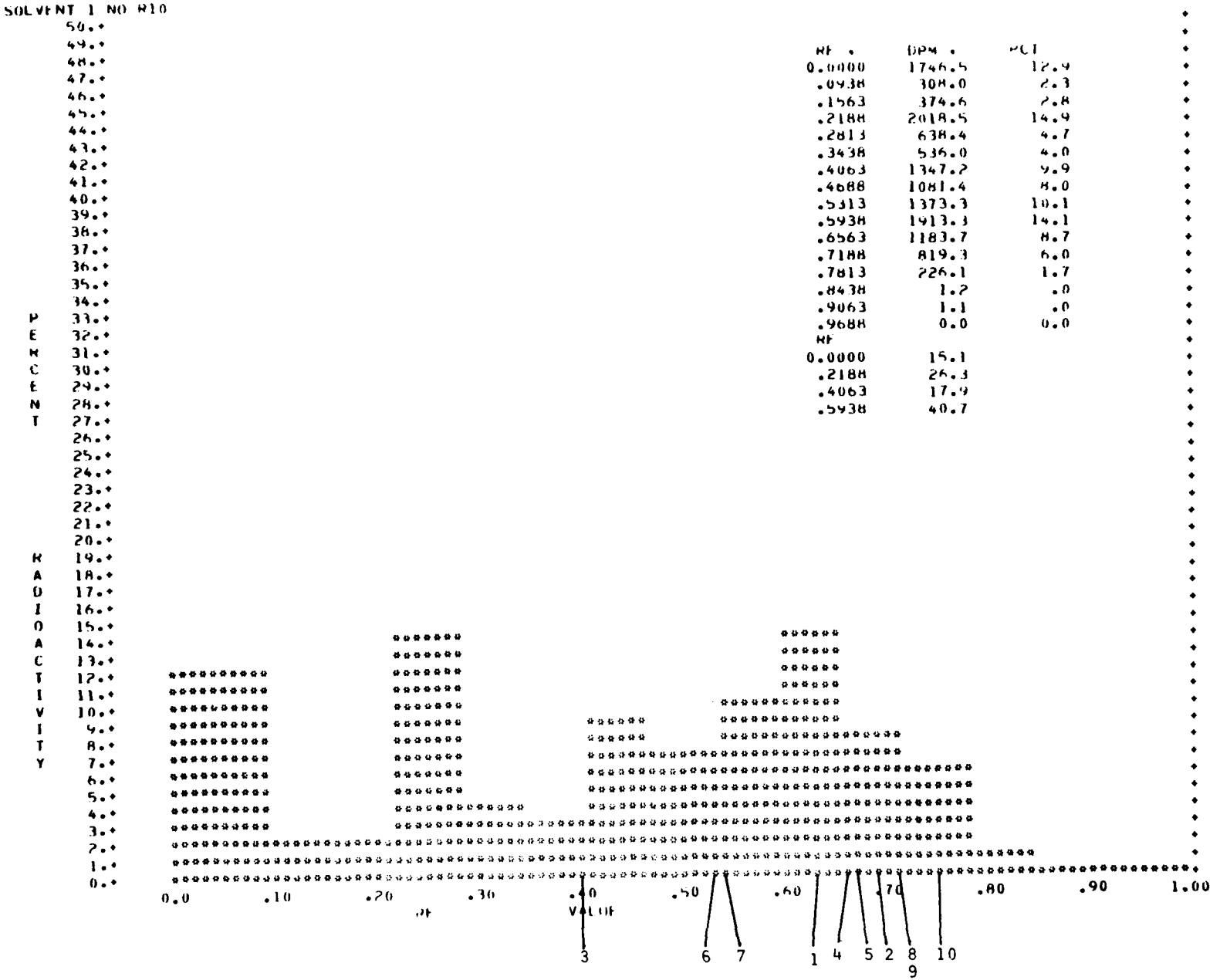

Figure 12-e-I: 4-Hr Urine, Male Rats, Oral Treatment, Solvent I

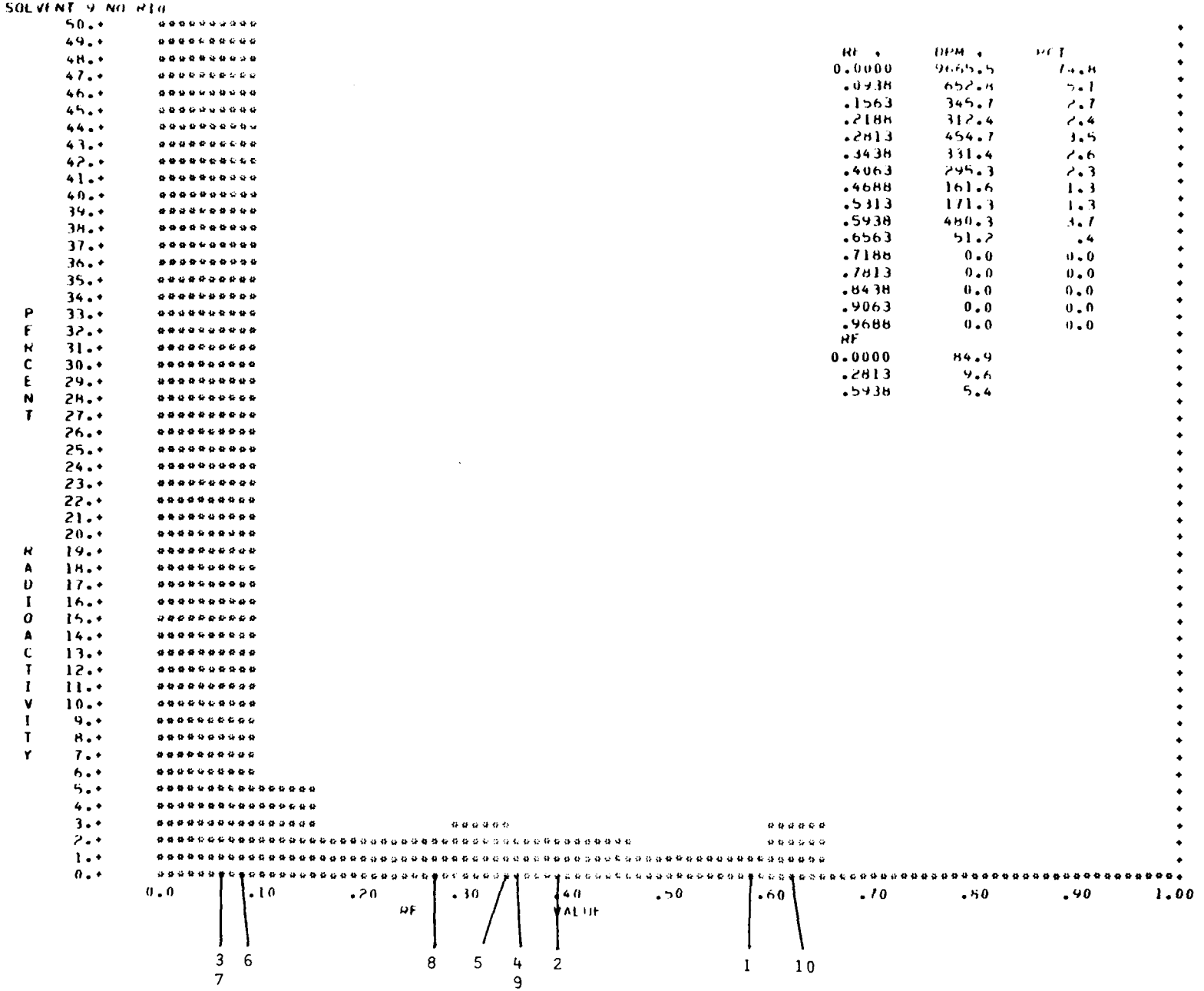

Figure 12-e-IX: 4-Hr Urine, Male Rats, Oral Treatment, Solvent IX

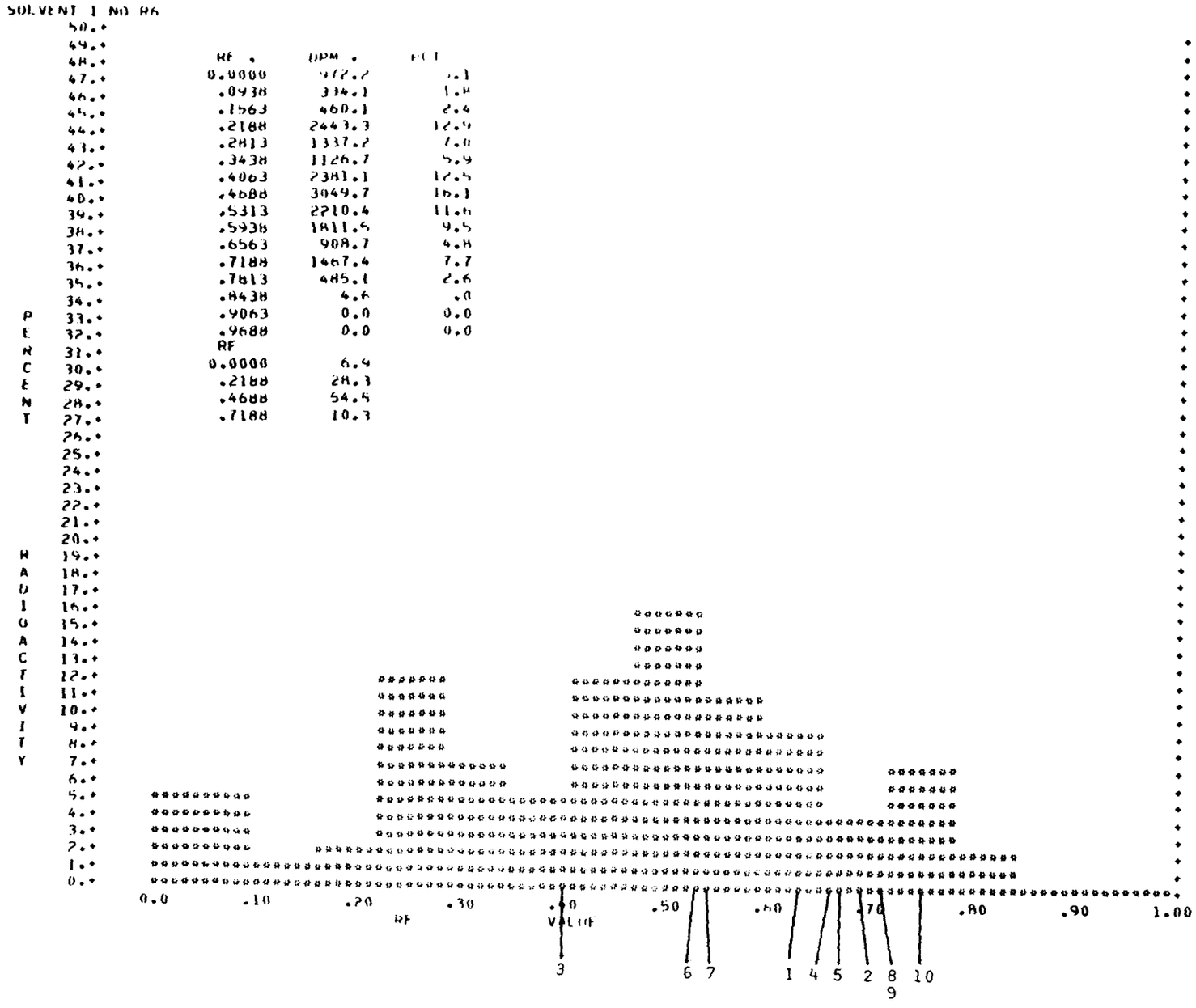

Figure 12-f-I: 4-Hr Urine, Male Rats, Intratracheal Instillation, Solvent I

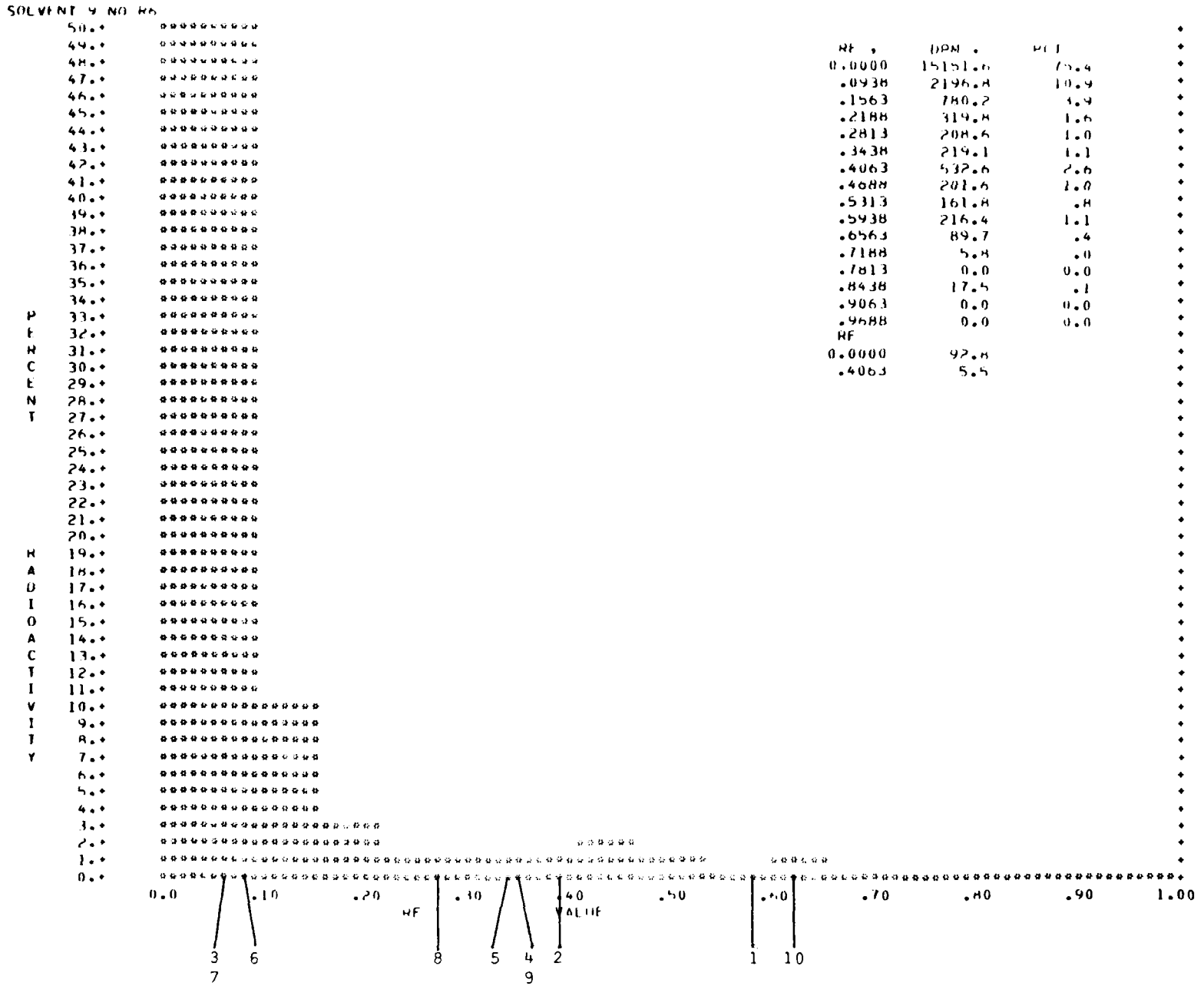

Figure 12-f-IX: 4-Hr Urine, Male Rats, Intratracheal Instillation, Solvent IX

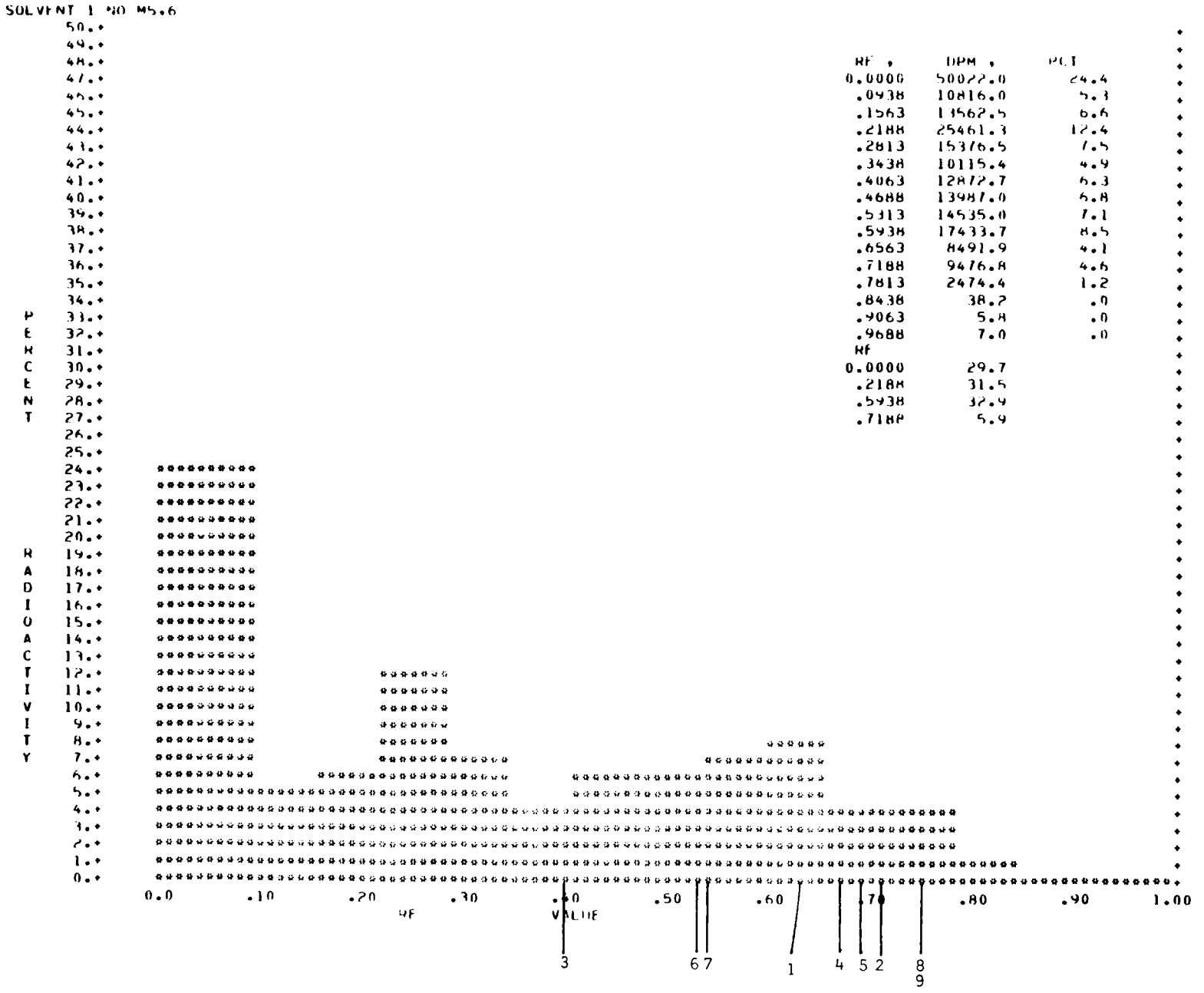

Figure 12-g-I: 24-Hr Urine, Male Mice, Oral Treatment, Solvent I

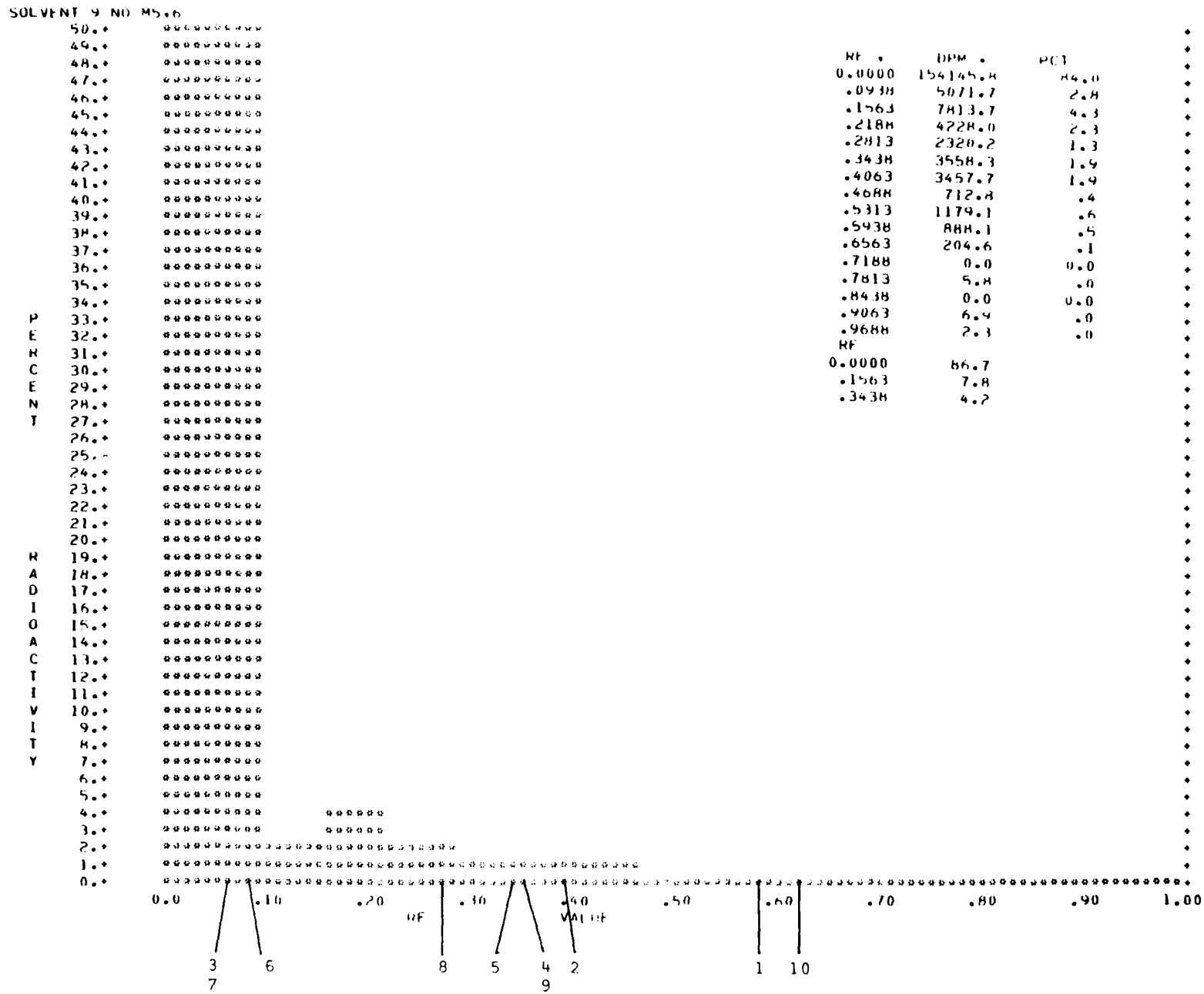

Figure 12-g-IX: 24-Hr Urine, Male Mice, Oral Treatment, Solvent IX

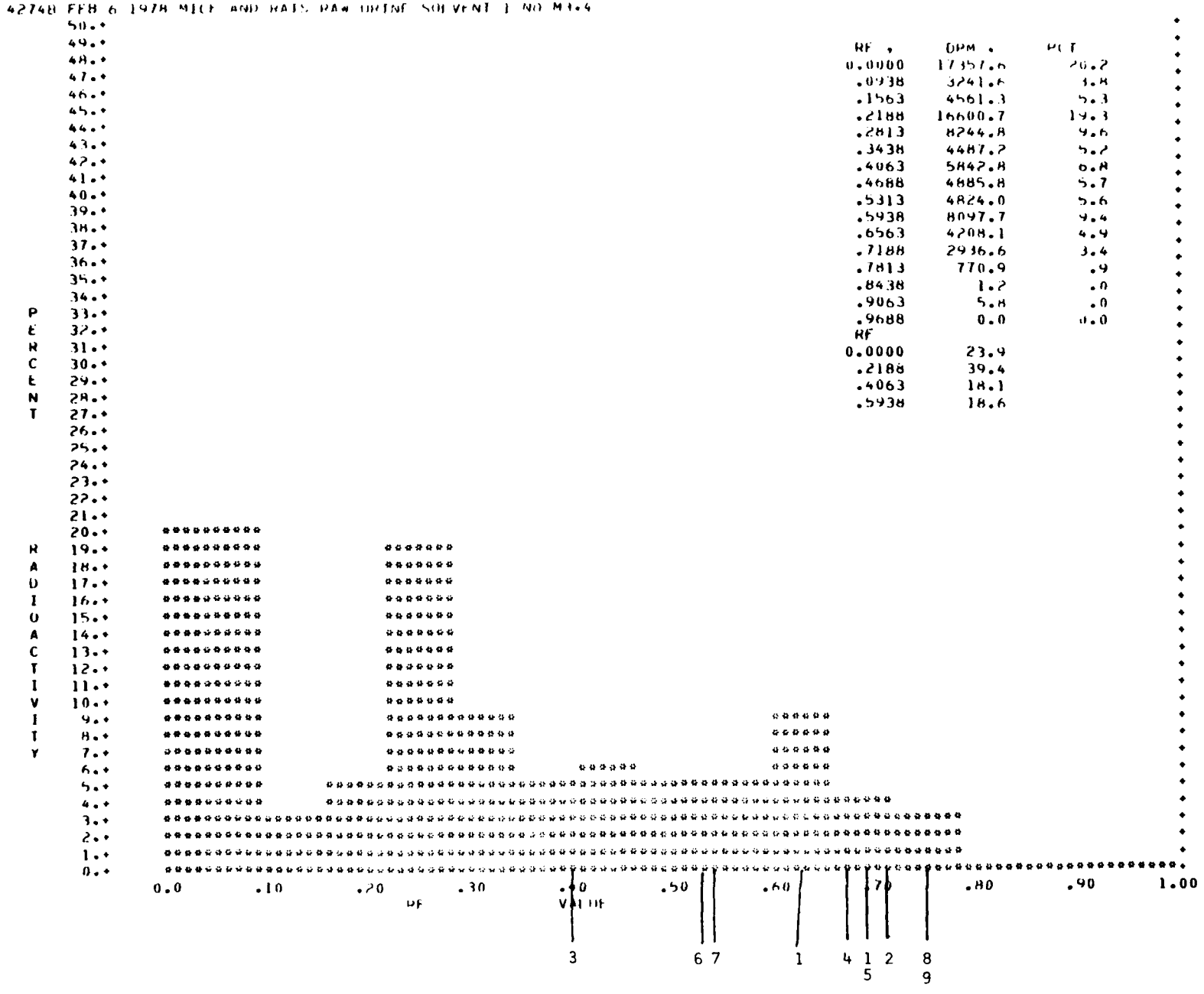

Figure 12-h-I: 24-Hr Urine, Male Mice, Dermal Application, Solvent I

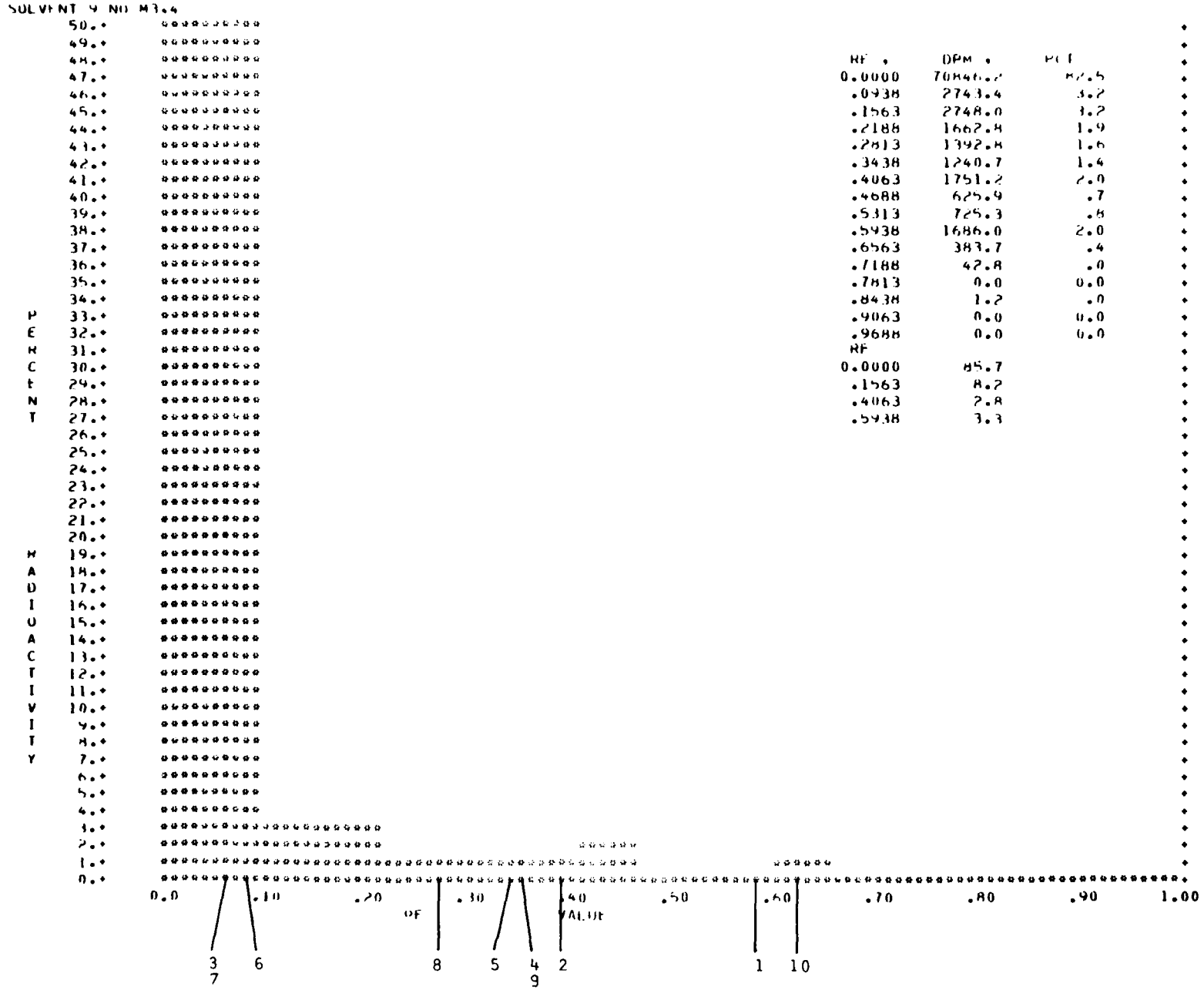

Figure 12-h-IX: 24 Hr Urine, Male Mice, Dermal Application, Solvent IX

Figure 13: TLC of Lyophilized Urine Obtained from Rats, Mice and Rabbits Treated Orally or Dermally with ^{14}C-TNT. Reference standards are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzylalcohol
3. Trinitrobenzoic Acid
4. 4-Amino-2,6-Dinitrotoluene
5. 2-Amino-4,6-Dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2,6'-Tetranitro-4,4'-azoxytoluene

Figure 13 follows

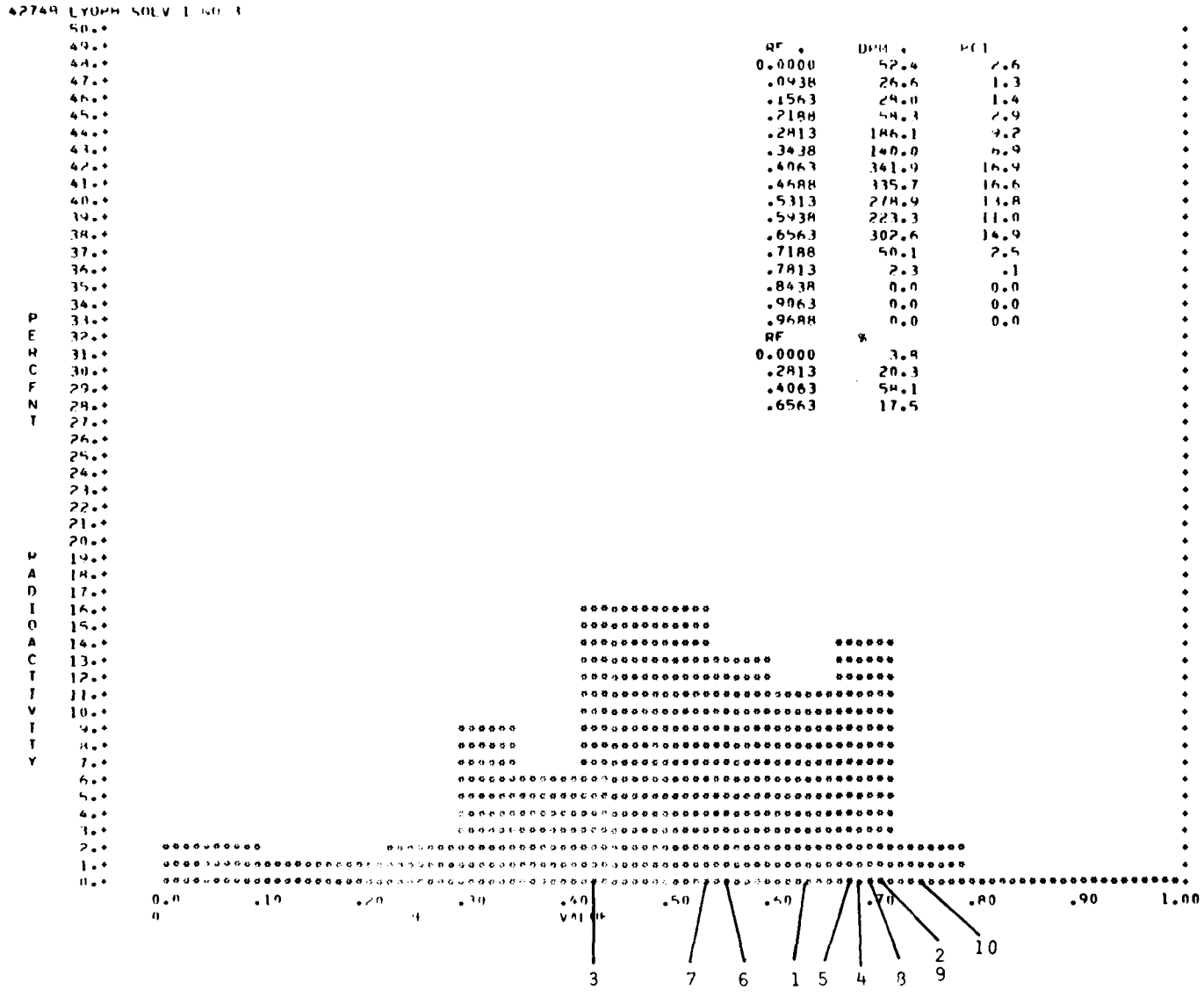

Figure 13-a-I: Male Rats, Oral Treatment, Solvent I

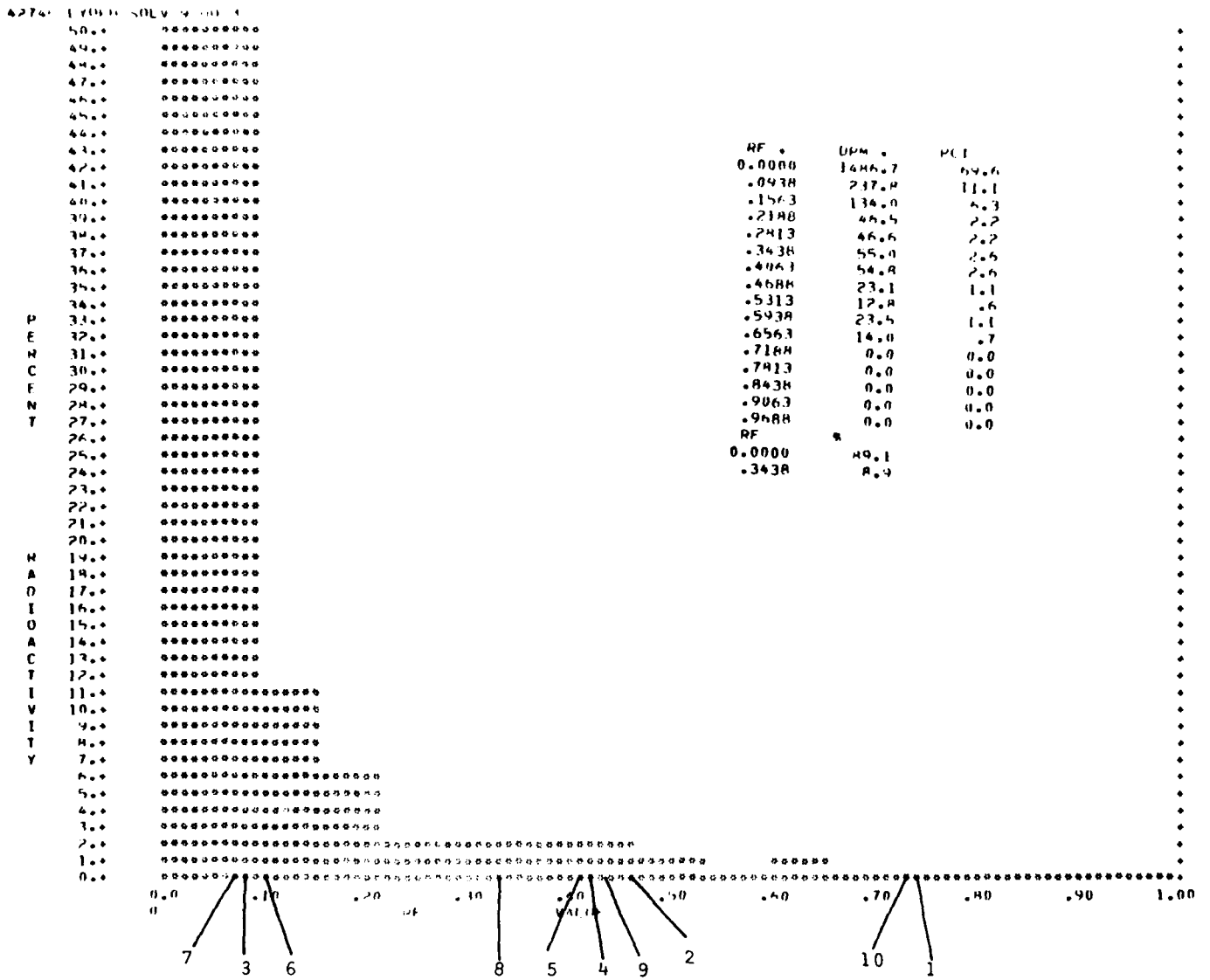

Figure 13-a-IX: Male Rats, Oral Treatment, Solvent IX

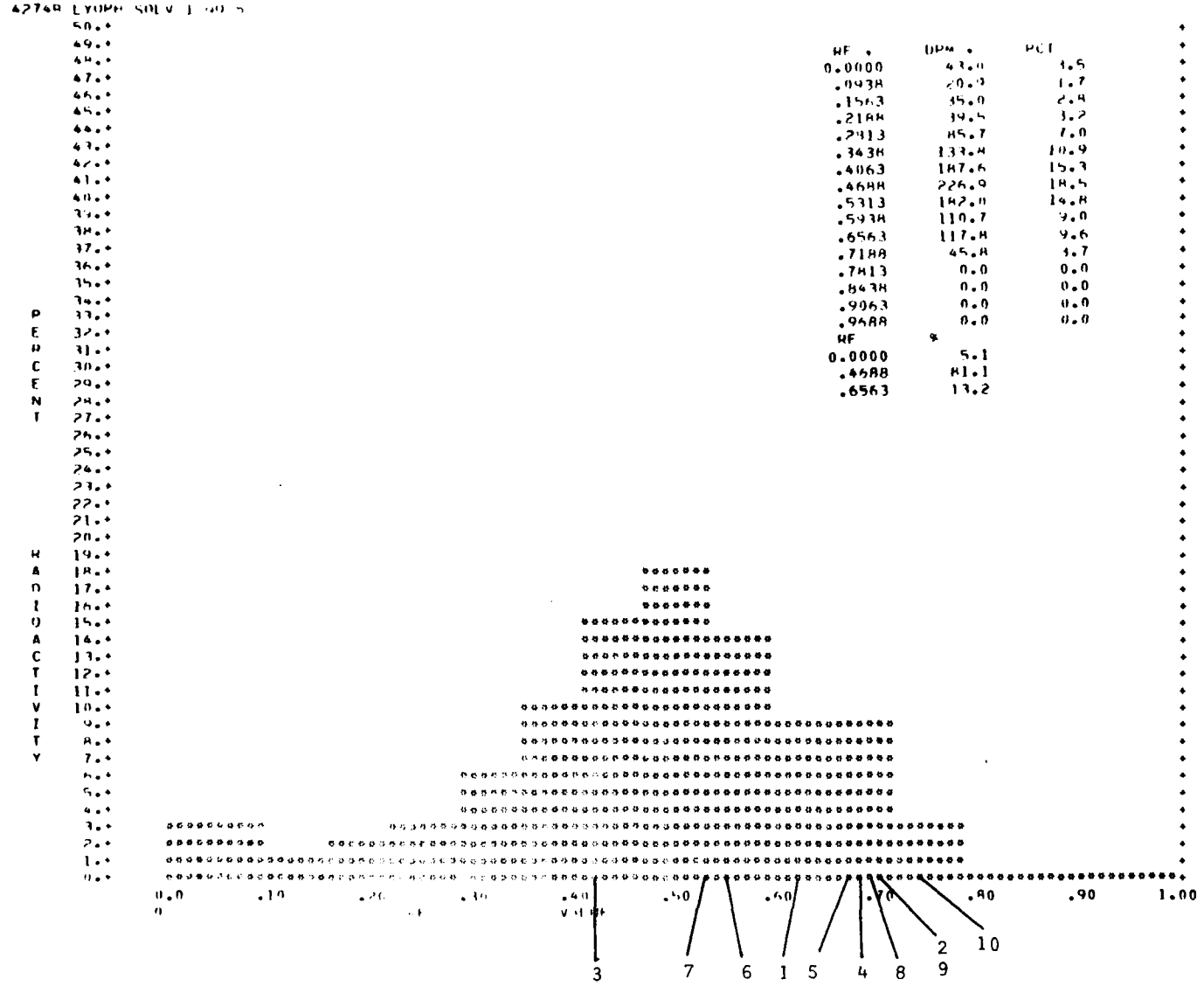

Figure 13-b-I: Female Rats, Oral Treatment, Solvent I

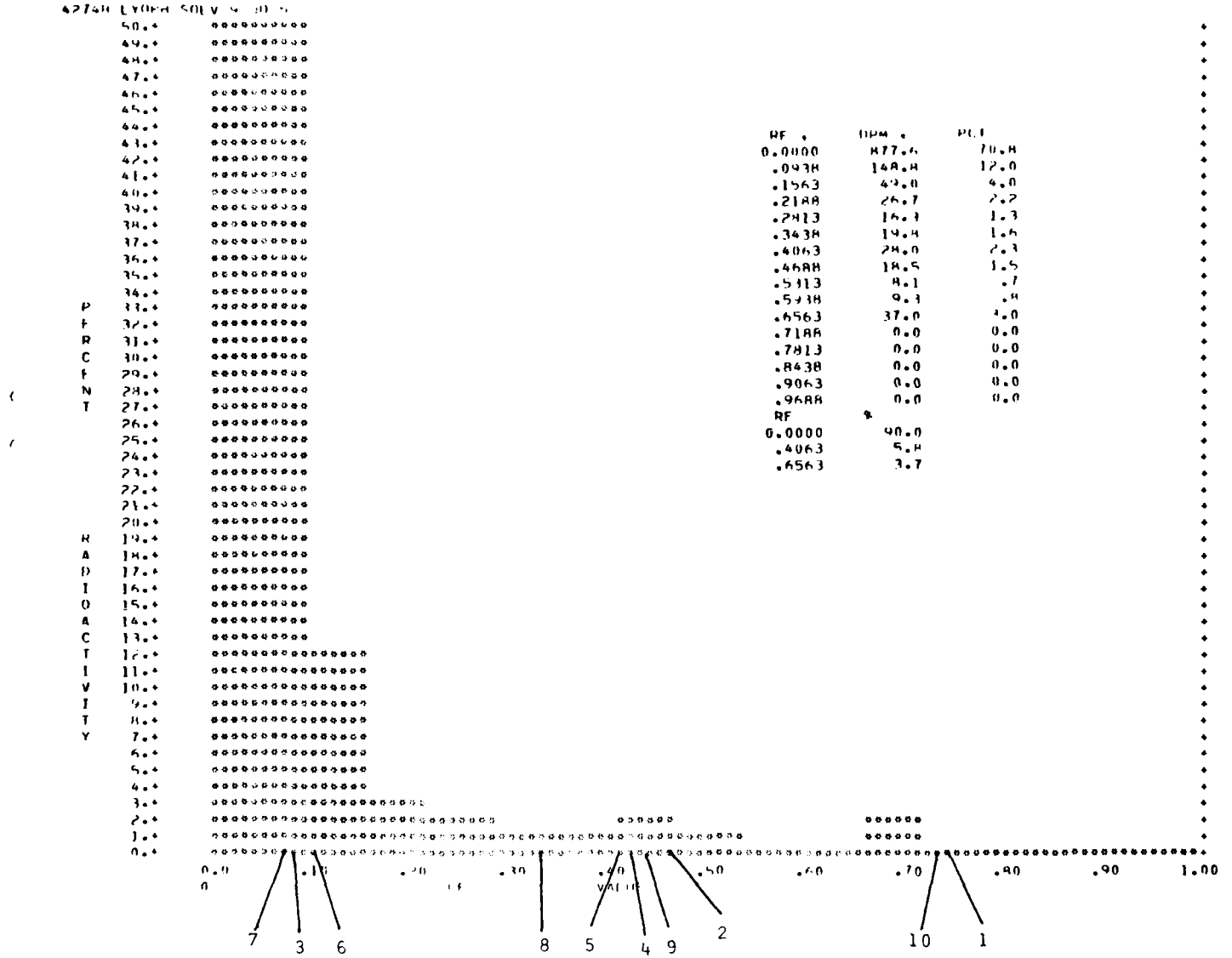

Figure 13-b-IX: Female Rats, Oral Treatment, Solvent IX

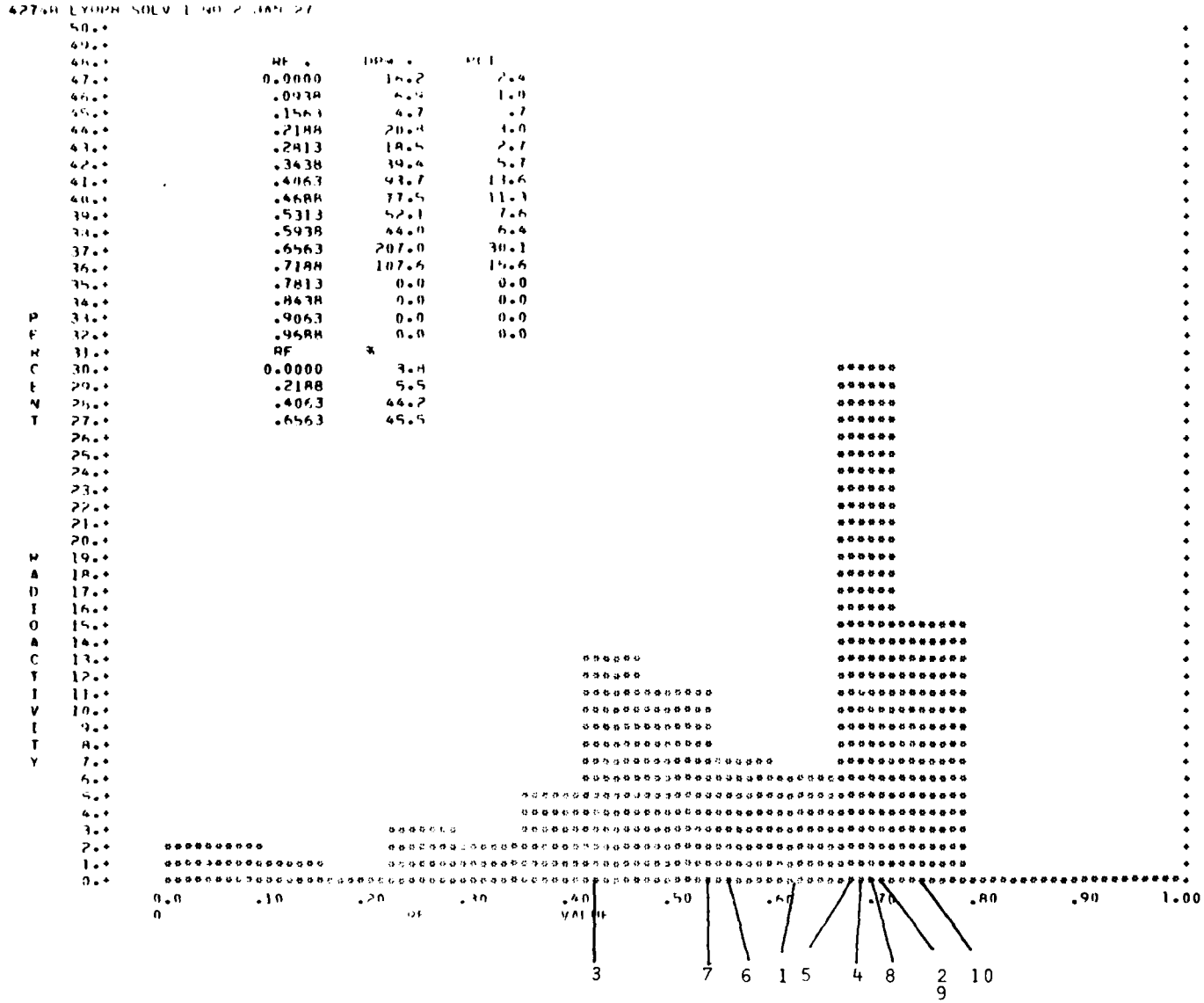

Figure 13-c-I: Male Rats, Dermal Application, Solvent I

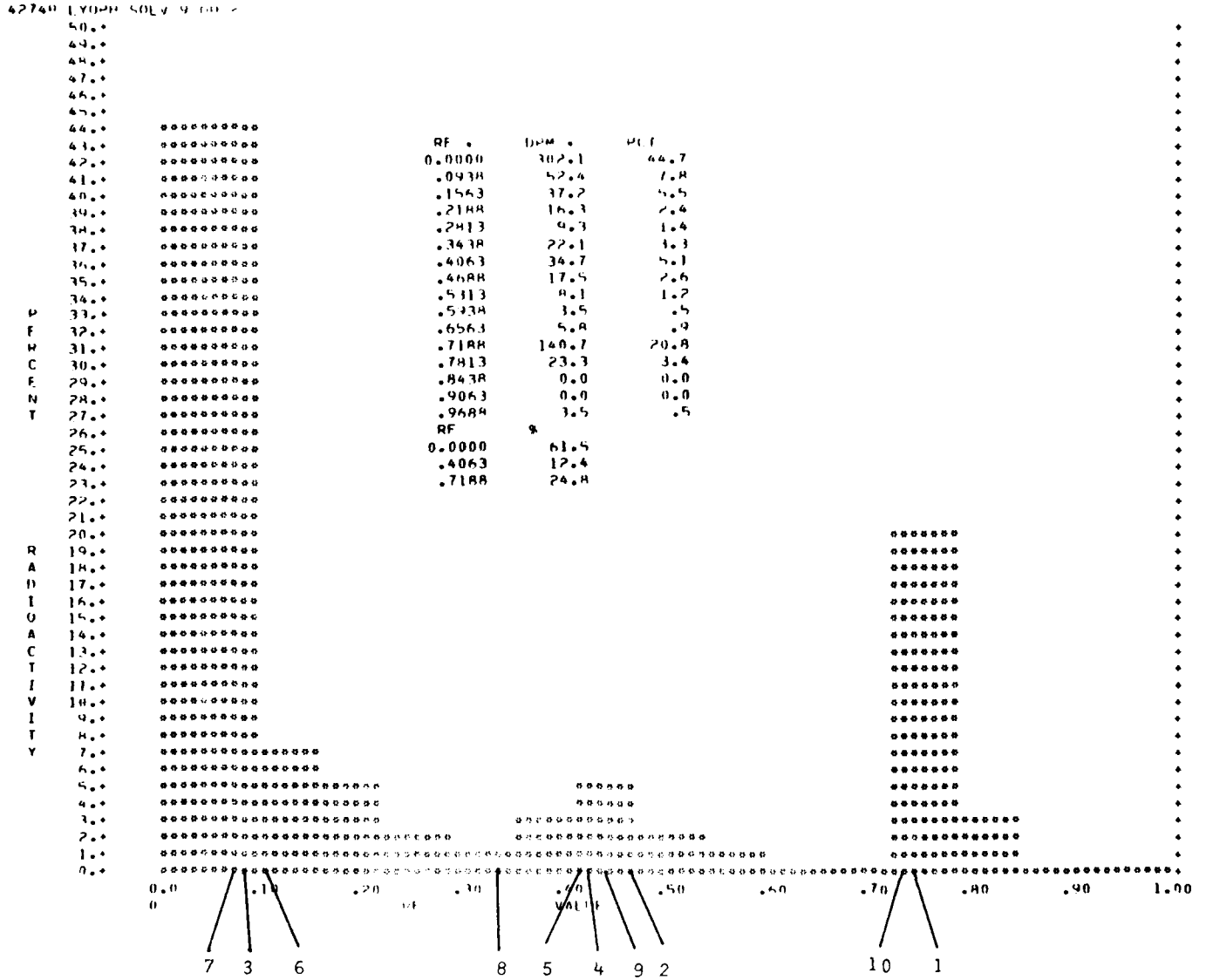

Figure 13-c-IX: Male Rats, Dermal Application, Solvent IX

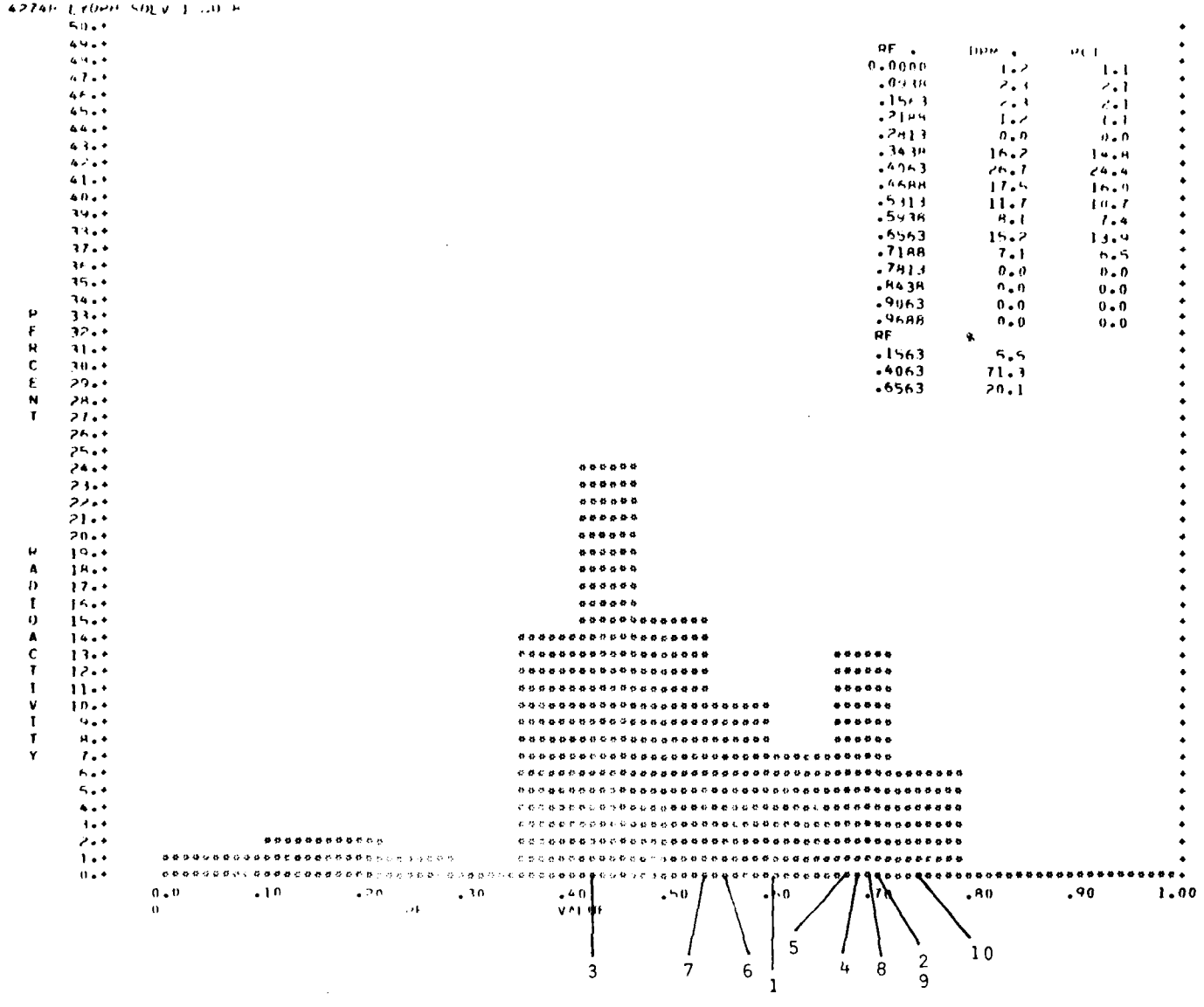

Figure 13-d-I: Female Rats, Dermal Application, Solvent I

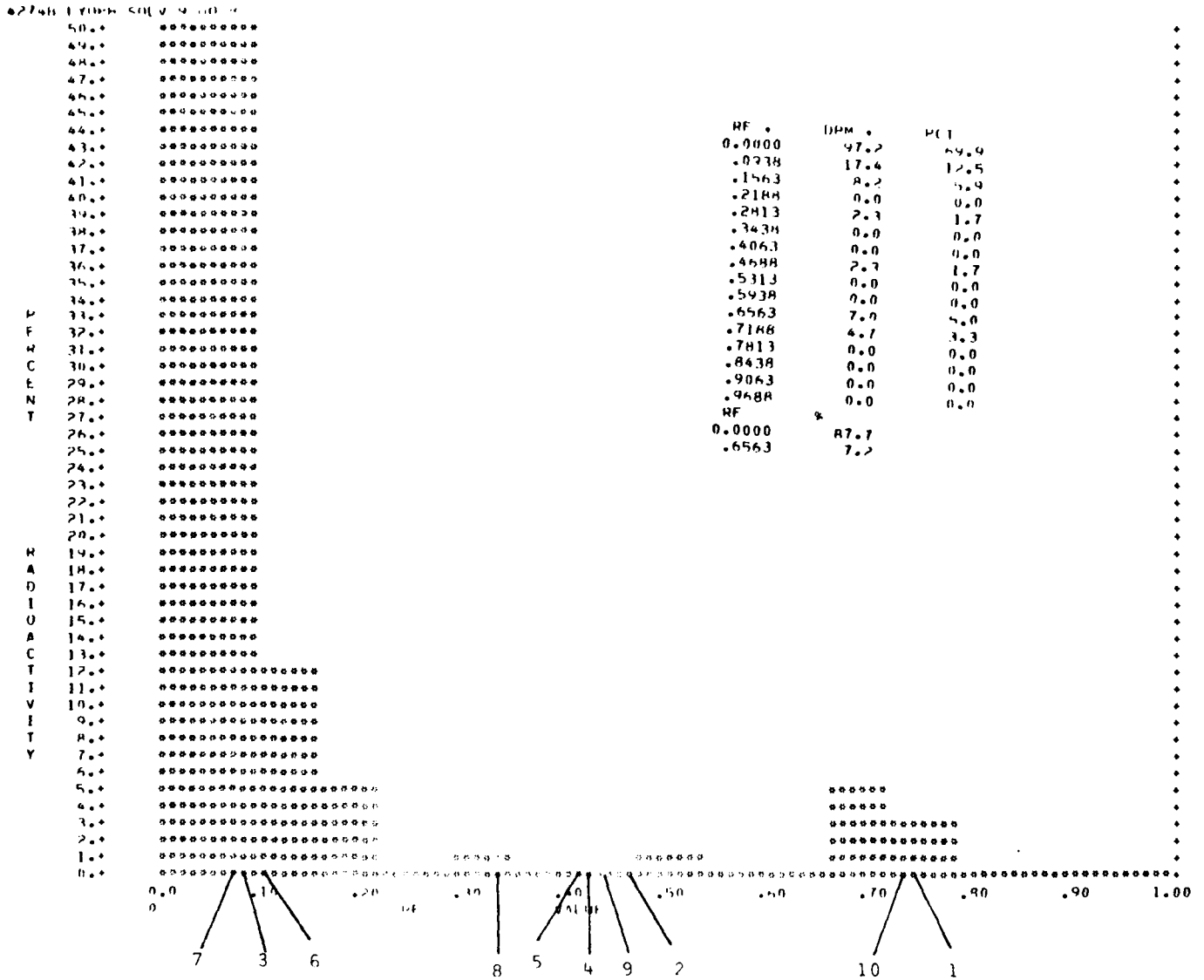

Figure 13-d-IX: Female Rats, Dermal Application, Solvent IX

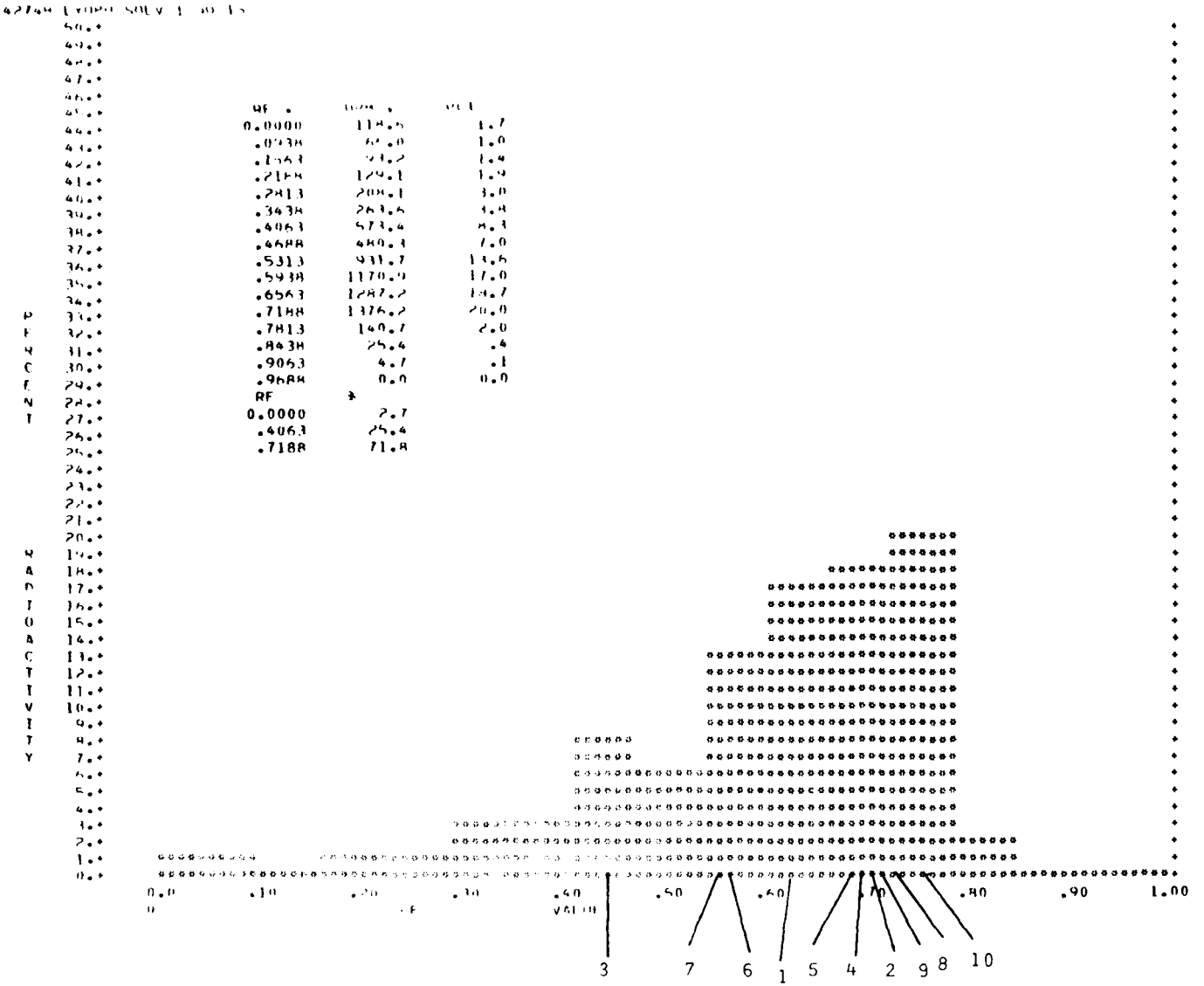

Figure 13-e-I: Male Mice, Oral Treatment, Solvent I

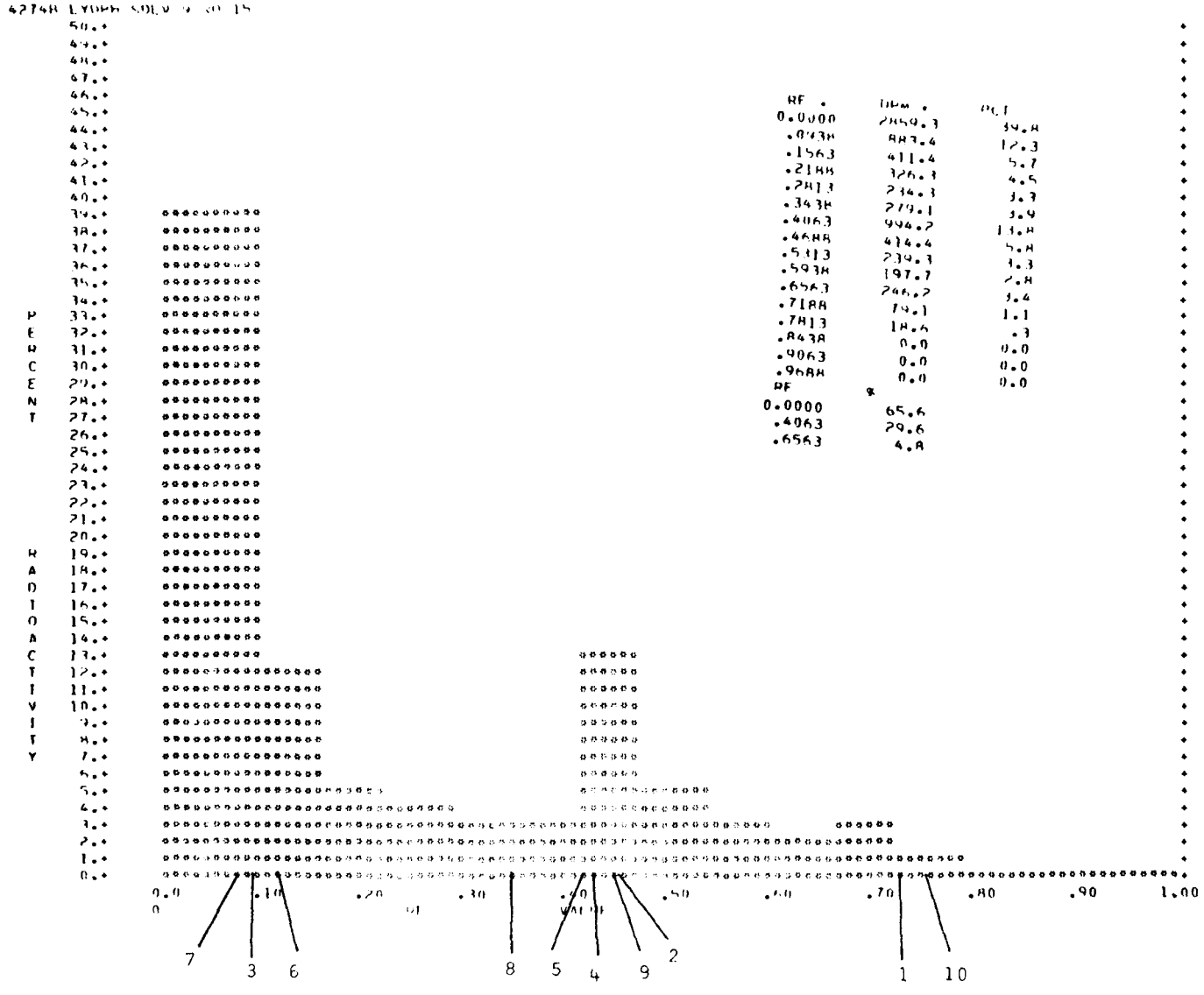

Figure 13-e-IX: Male Mice, Oral Treatment, Solvent IX

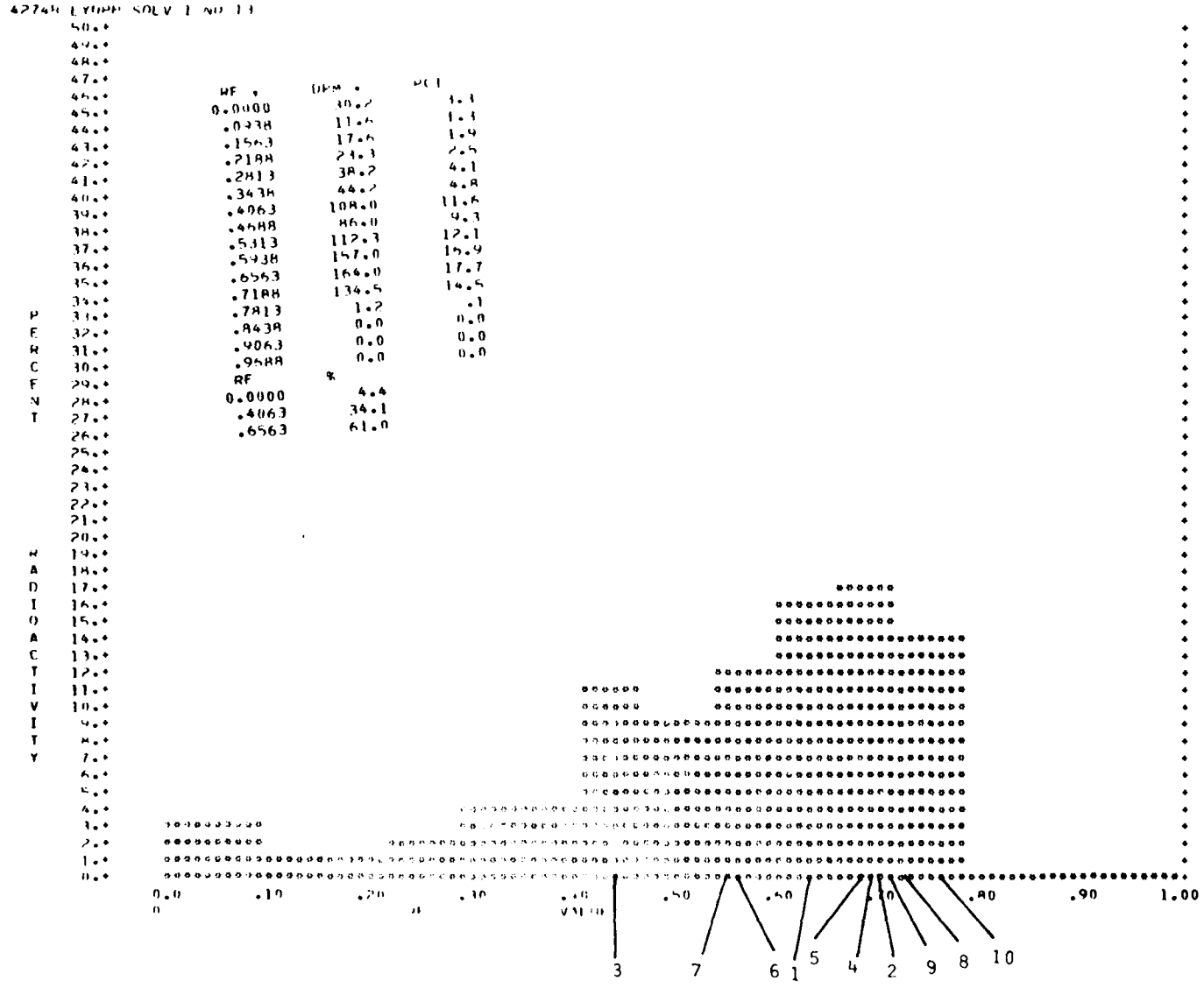

Figure 13-f-I: Male Mice, Dermal Application, Solvent I

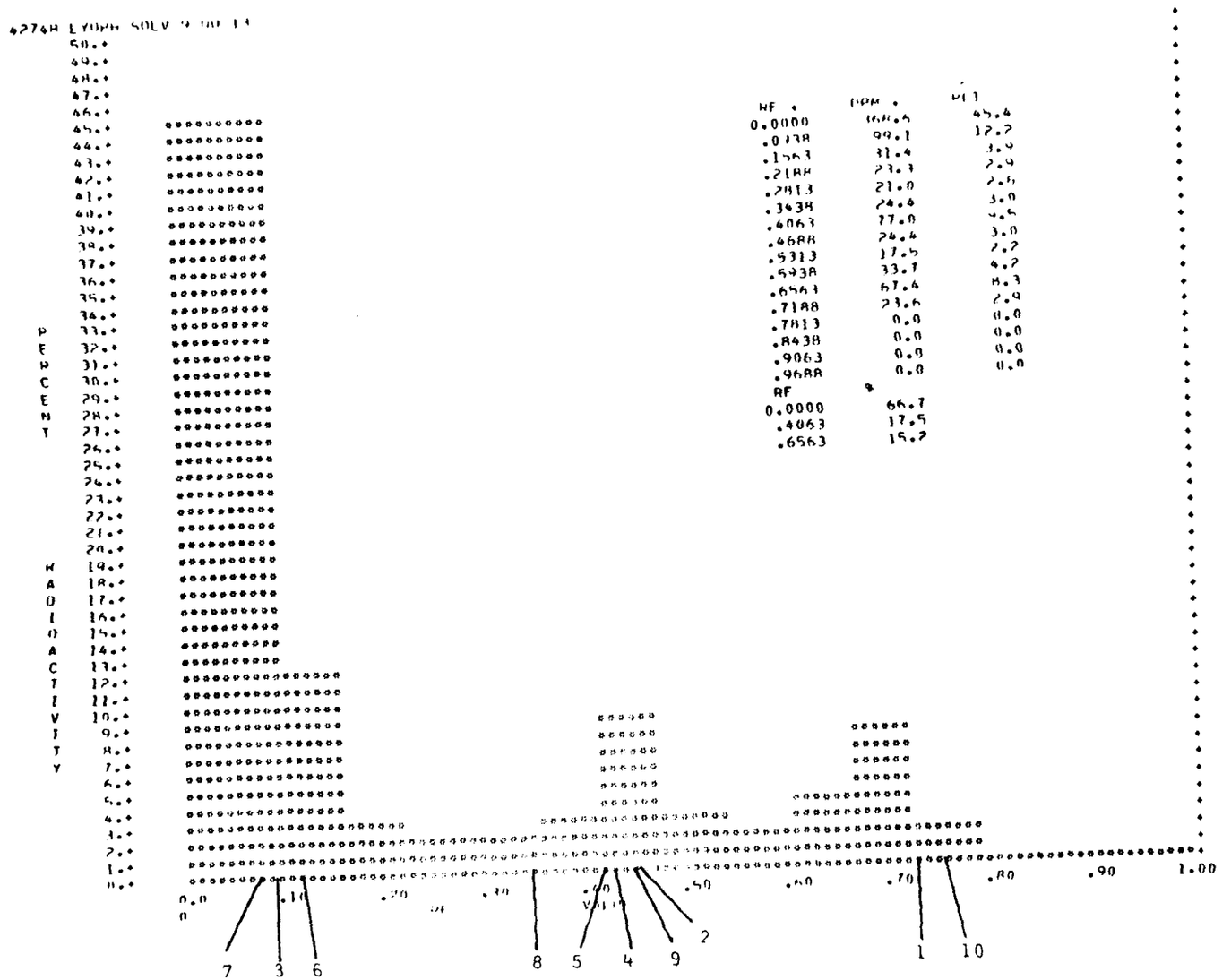

Figure 13-f-IX: Male Mice, Dermal Application, Solvent IX

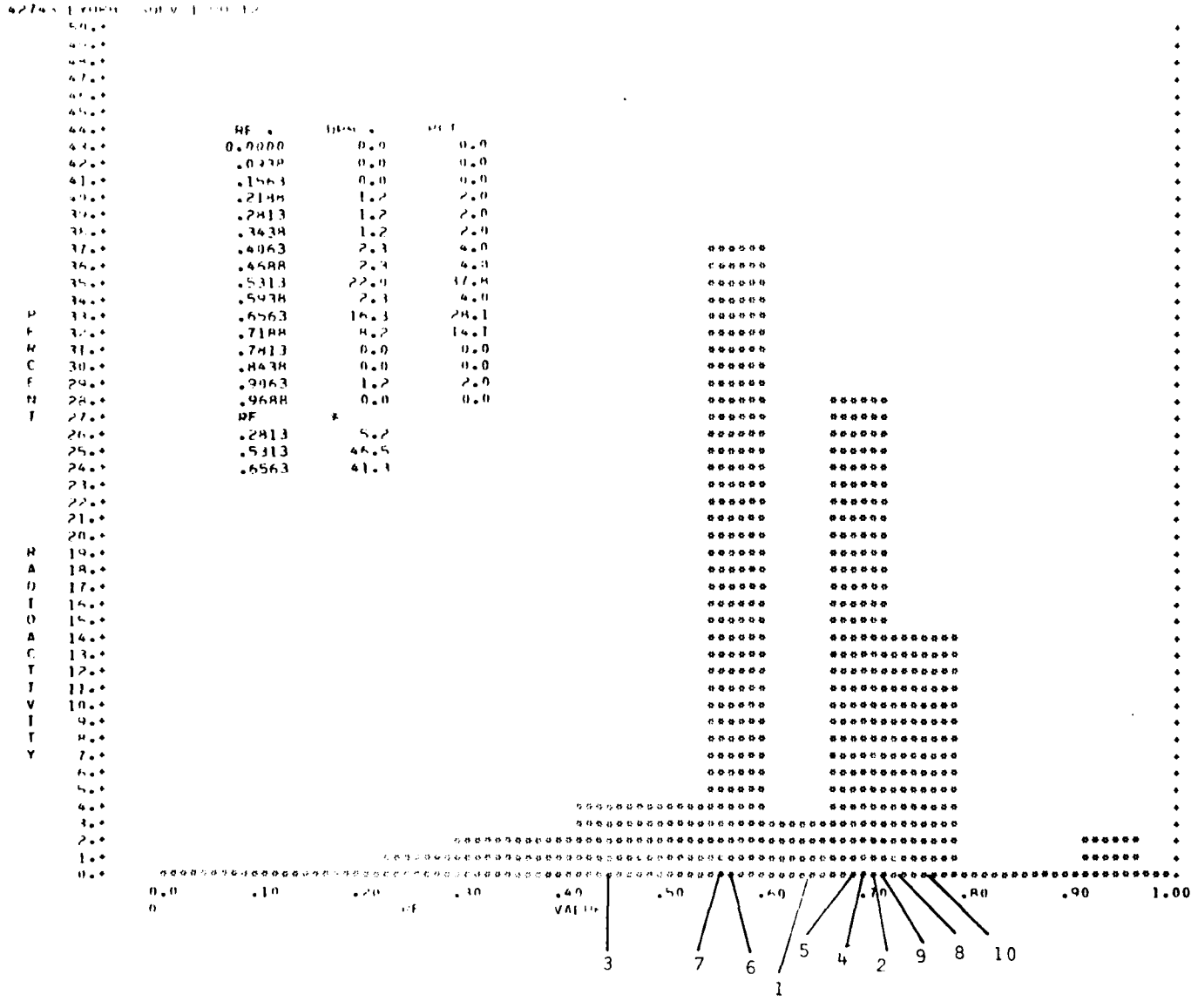

Figure 13-g-I: Male Rabbits, Oral Treatment, Solvent I

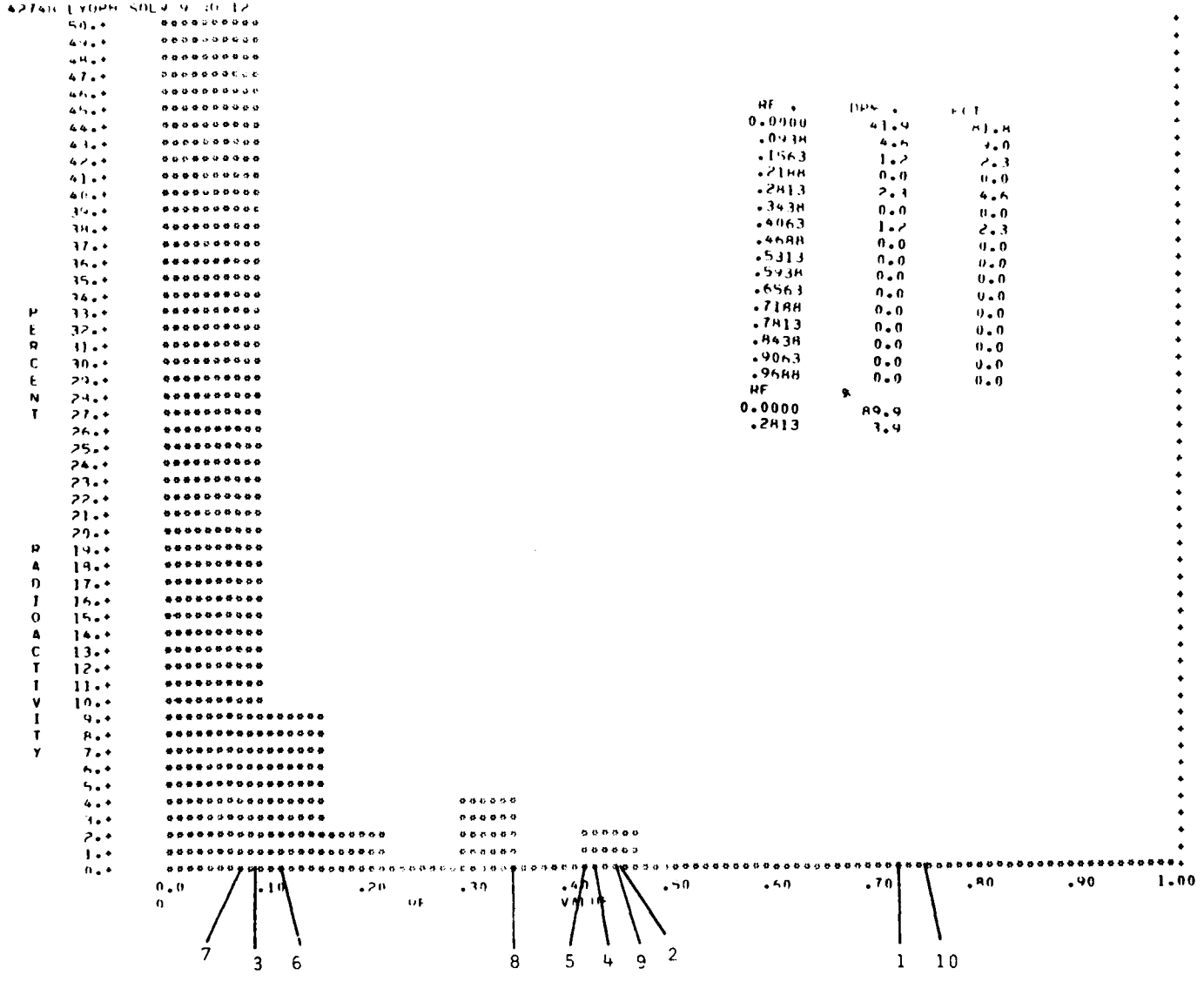

Figure 13-g-IX: Male Rabbits, Oral Treatment, Solvent IX

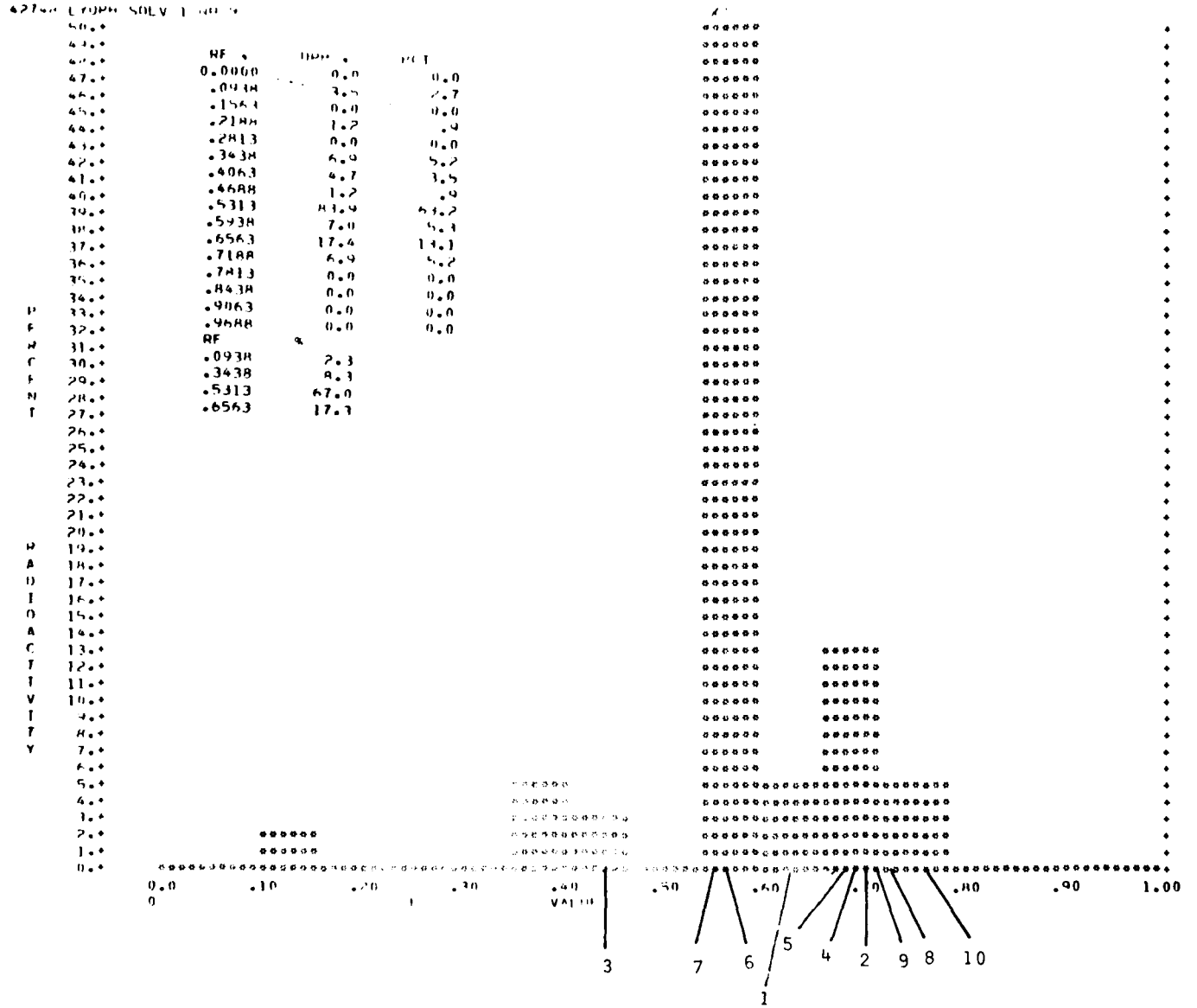

Figure 13-h-I: Male Rabbits, Dermal Application, Solvent I

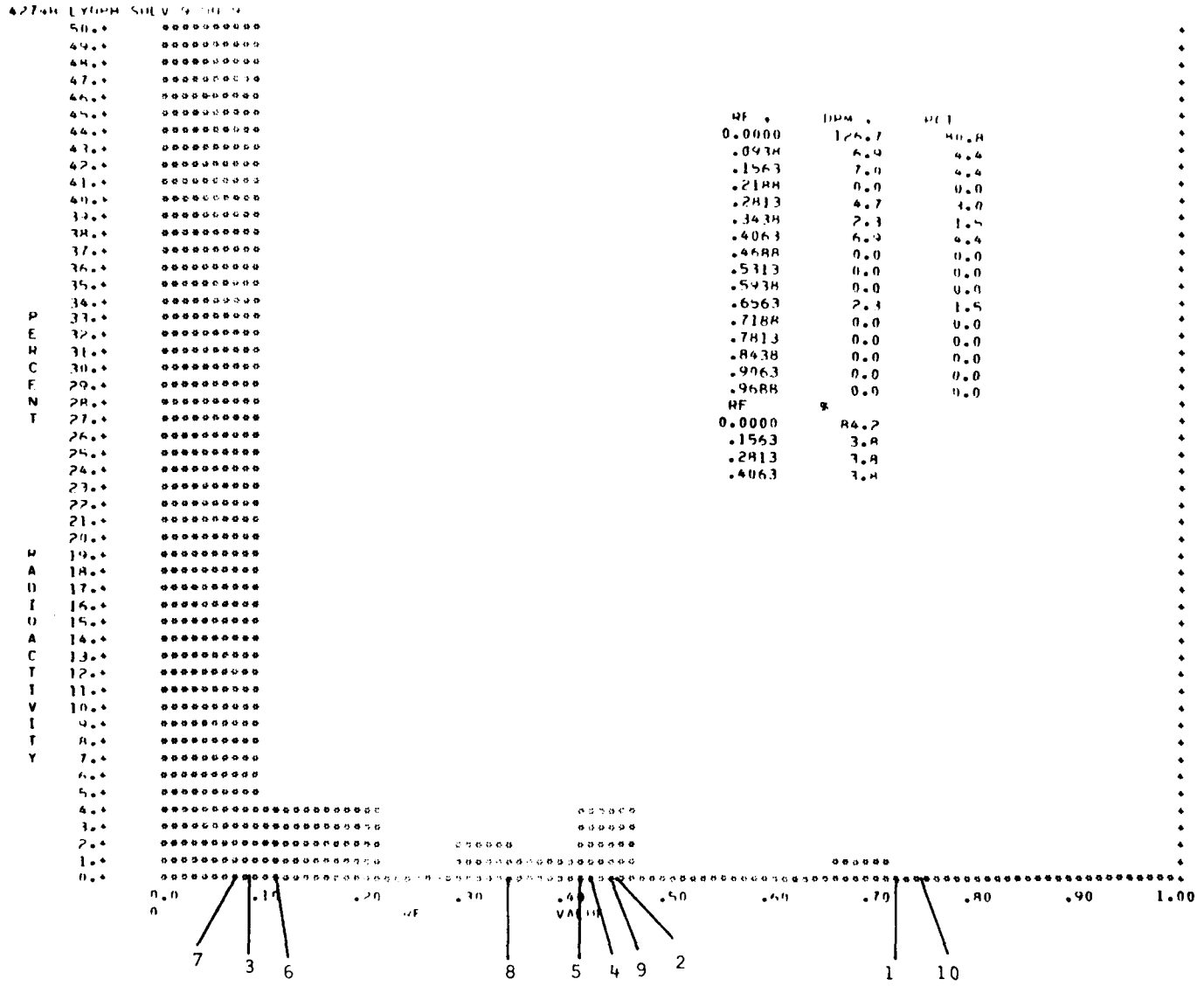

Figure 13-h-IX: Male Rabbits, Dermal Application, Solvent IX

Figure 14: TLC of Lyophilized Urine Obtained from Rats, Mice, Rabbits and Dogs Treated Orally or Dermally with ^{14}C-TNT. Plates were cut into 0.5 cm zones. Reference standards are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzylalcohol
3. Trinitrobenzoic Acid
4. 4-Amino-2,6-Dinitrotoluene
5. 2-Amino-4,6-Dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2,6'-Tetranitro-4,4'-azoxytoluene

Figure 14 follows

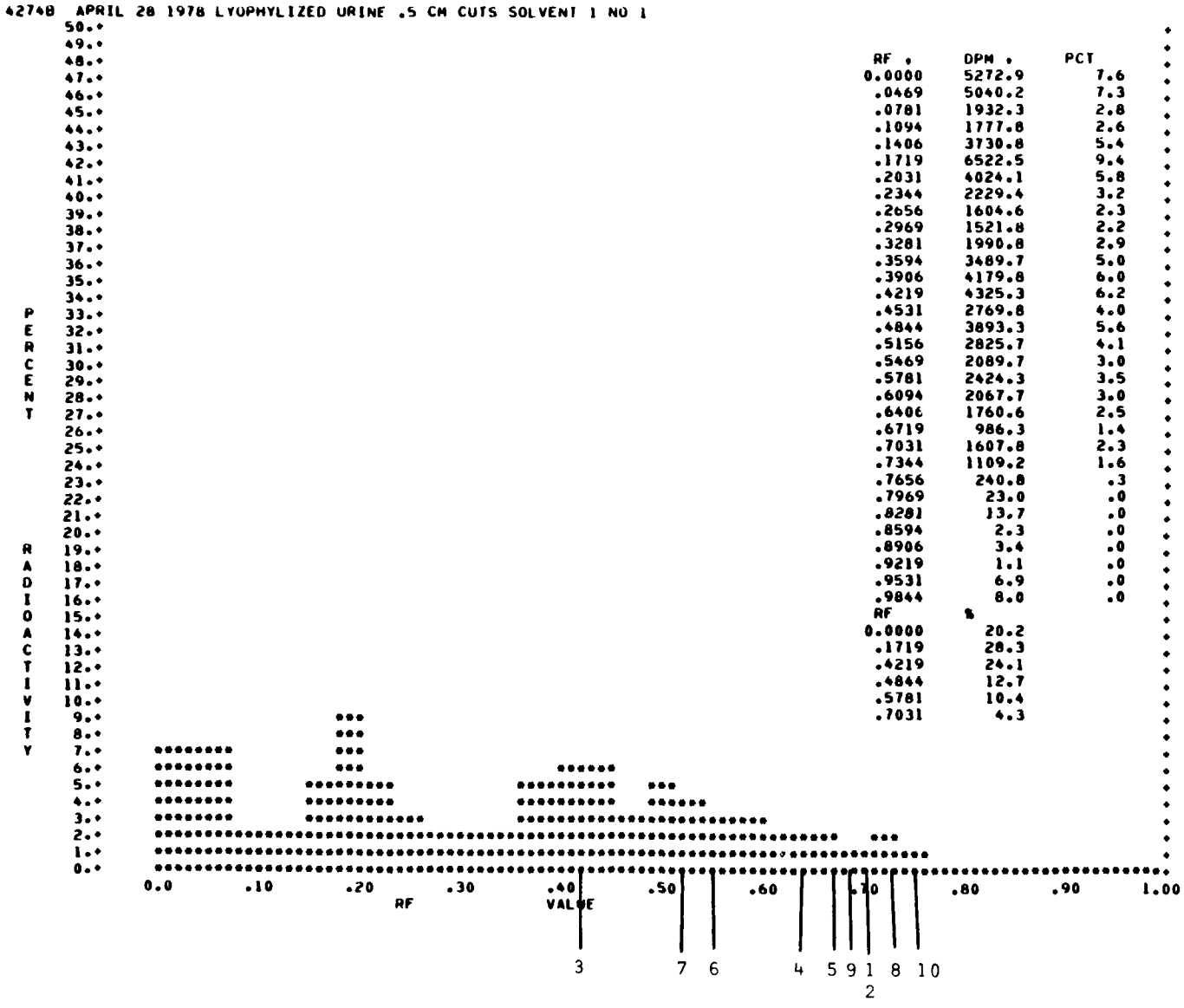

Figure 14-a-I: Male Rats, Oral Treatment, Solvent I

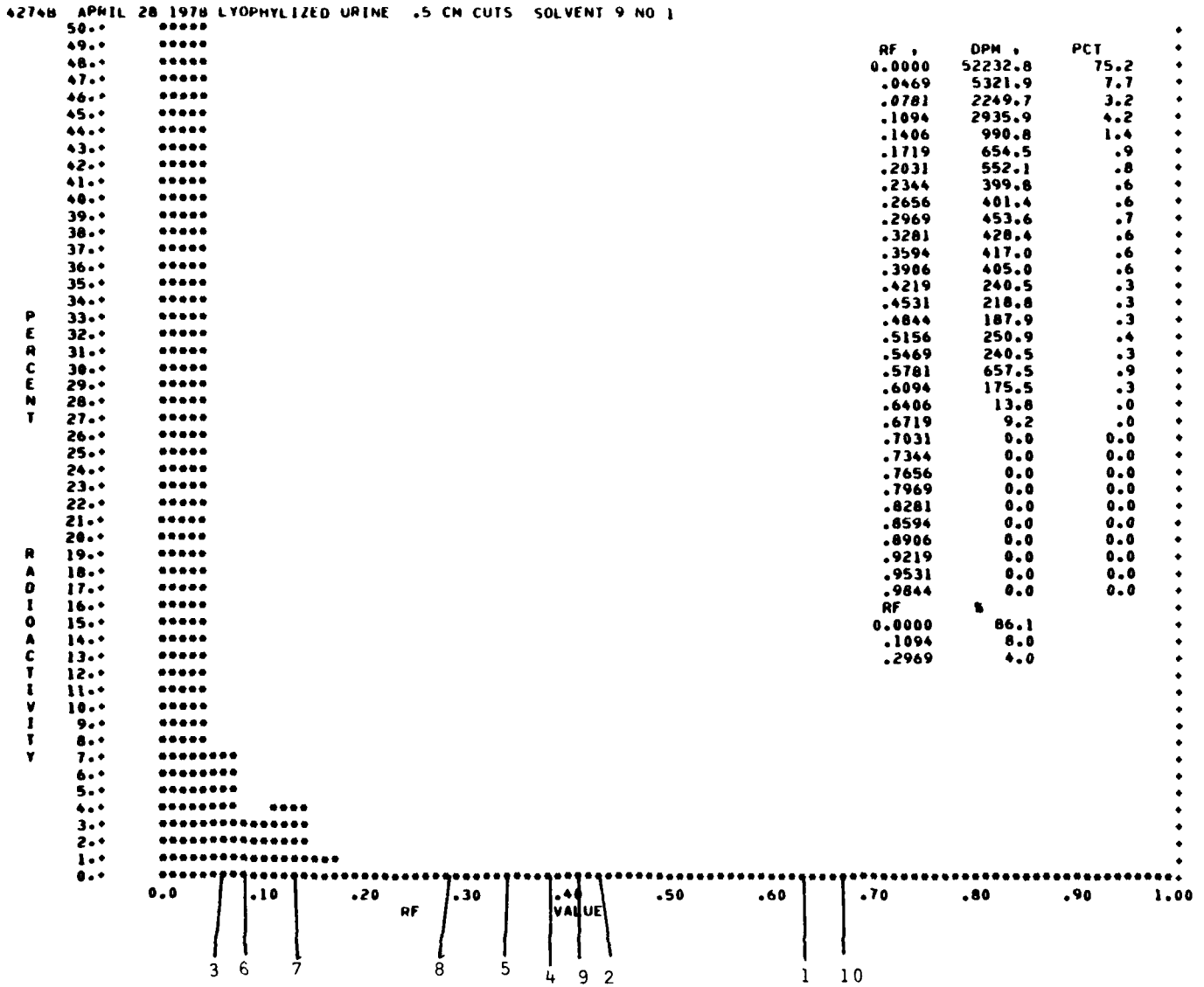

Figure 14-a-IX: Male Rats, Oral Treatment, Solvent IX

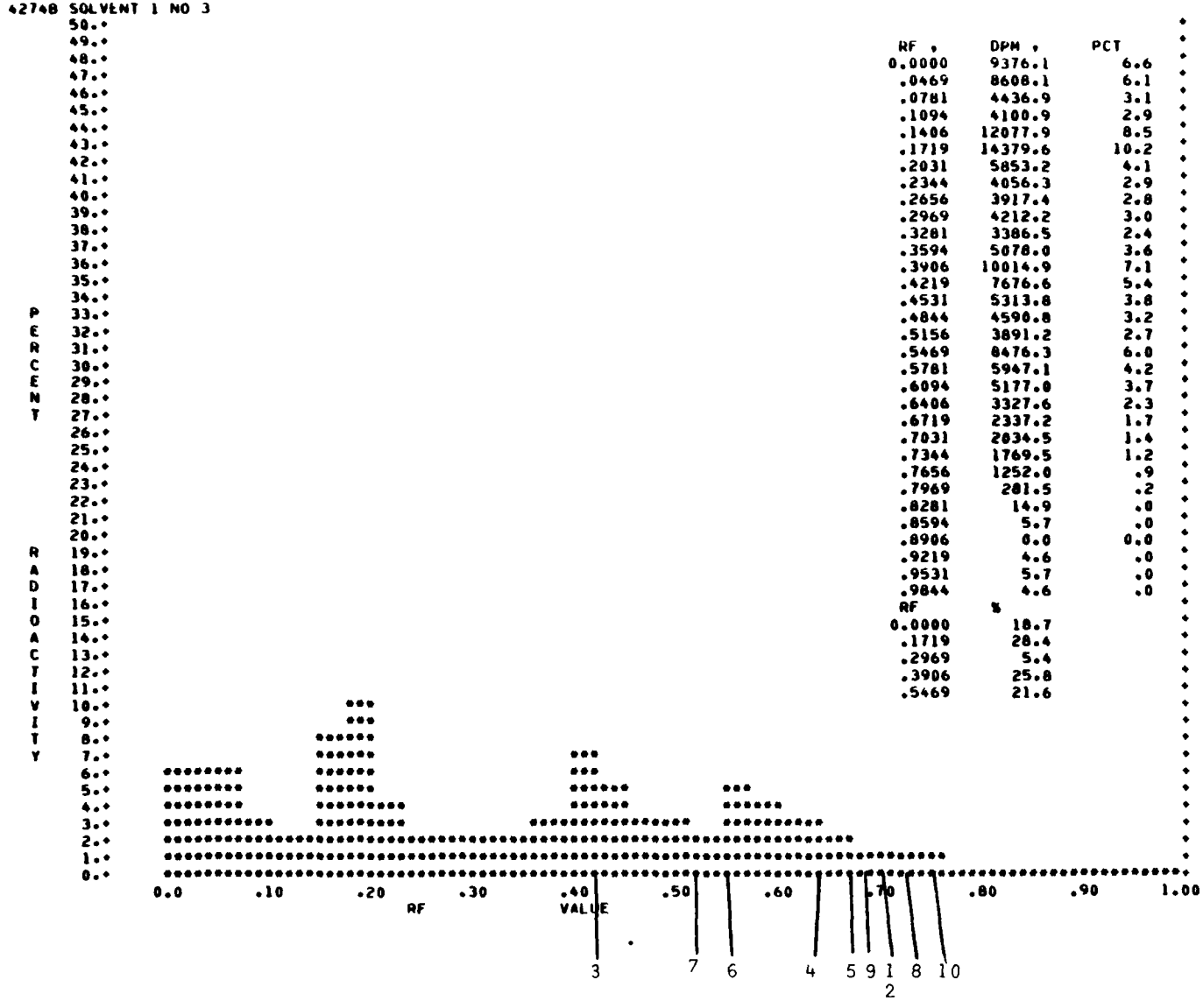

Figure 14-b-I: Female Rats, Oral Treatment, Solvent I

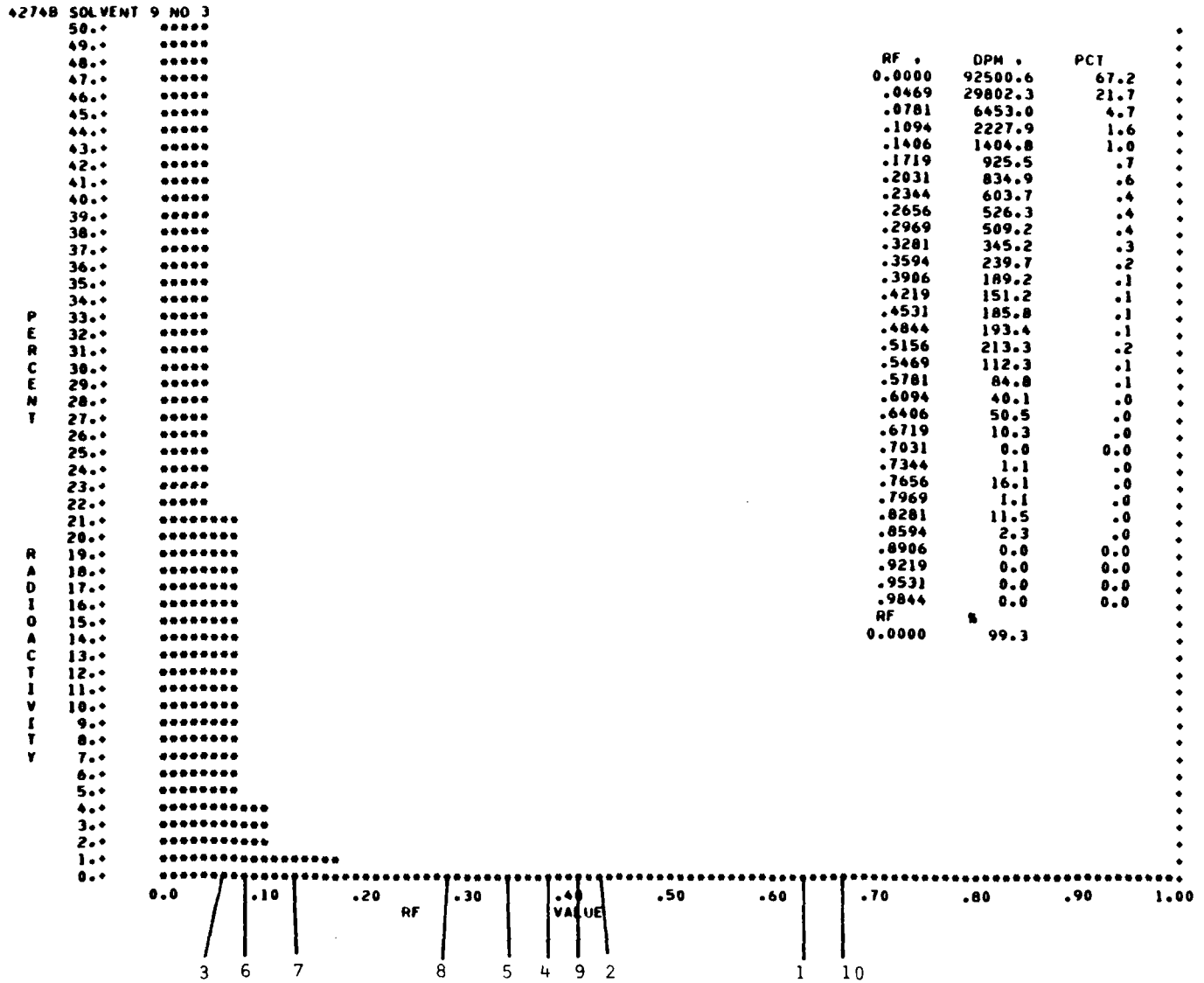

Figure 14-b-IX: Female Rats, Oral Treatment, Solvent IX

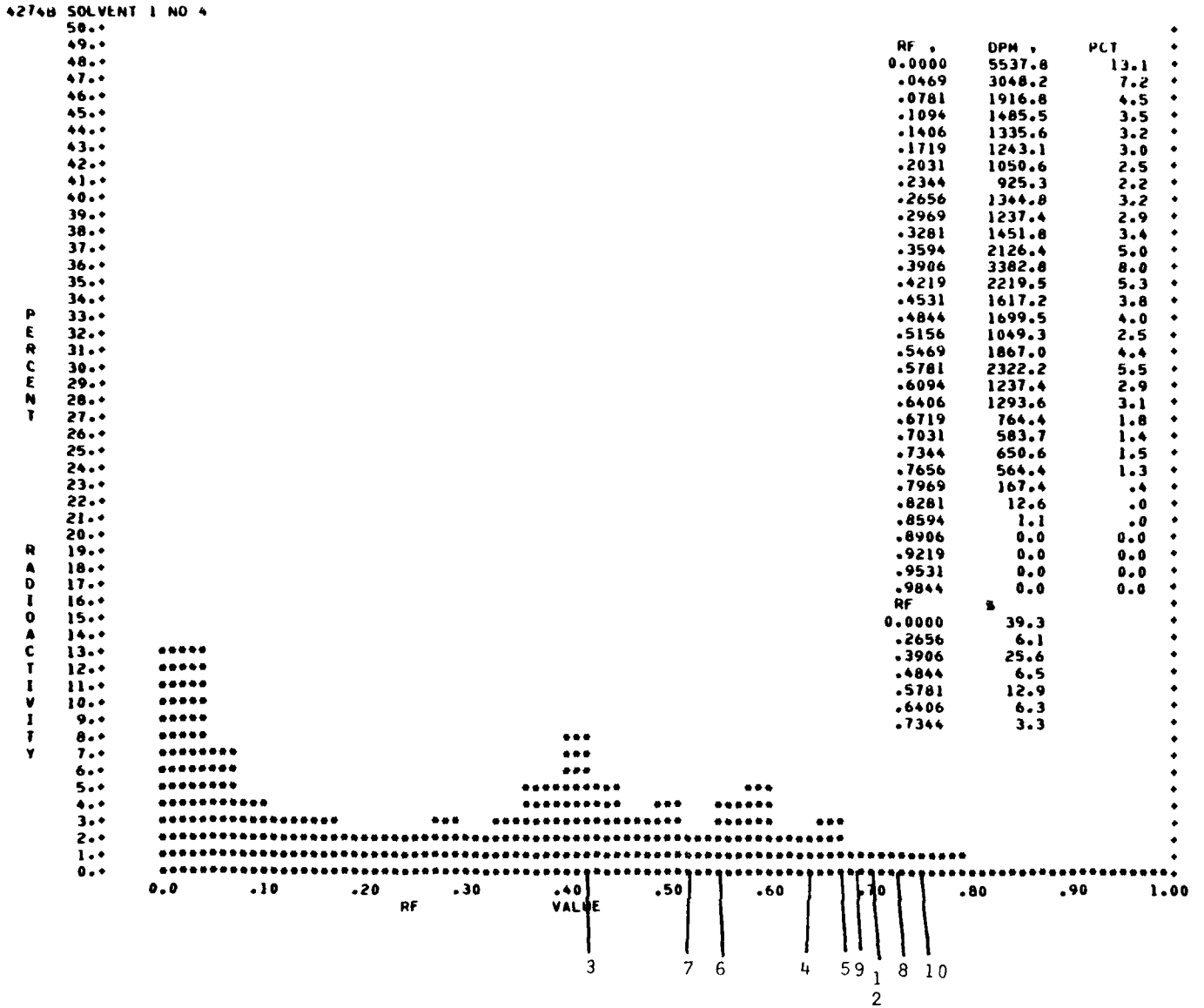

Figure 14-c-I: Male Rats, Dermal Application, Solvent I

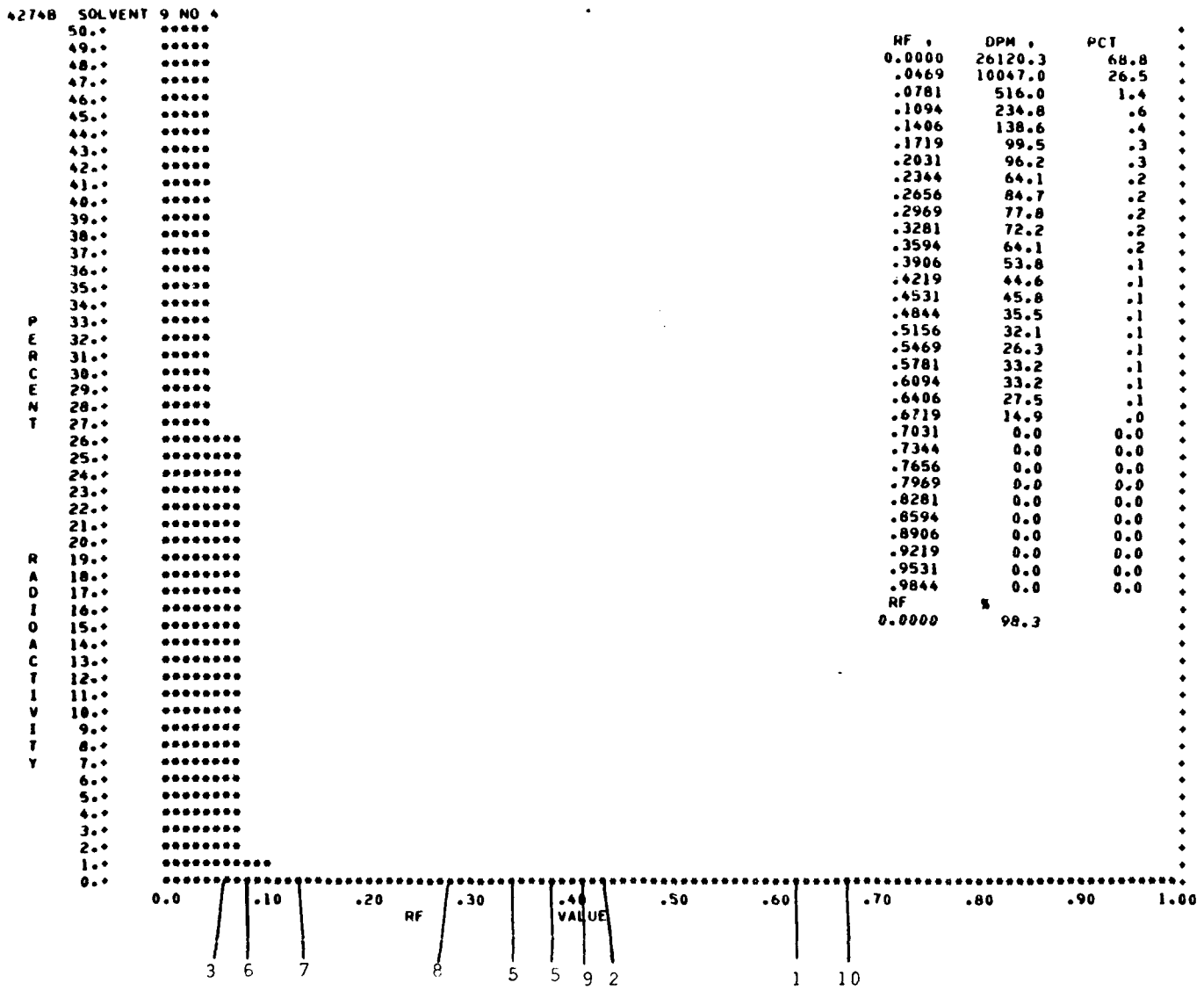

Figure 14-c-IX: Male Rats, Dermal Application, Solvent IX

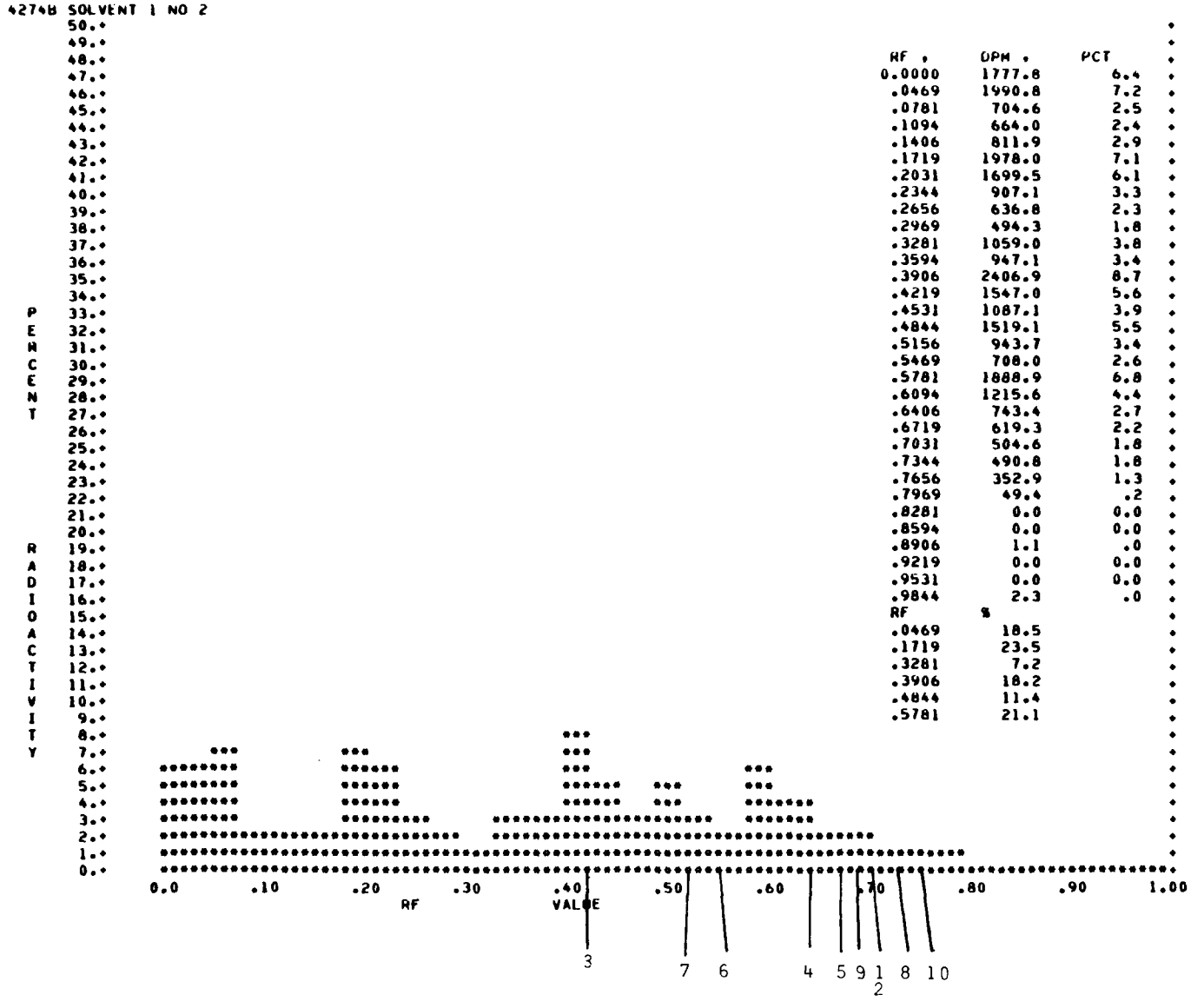

Figure 14-d-I: Female rats, Dermal Application, Solvent I

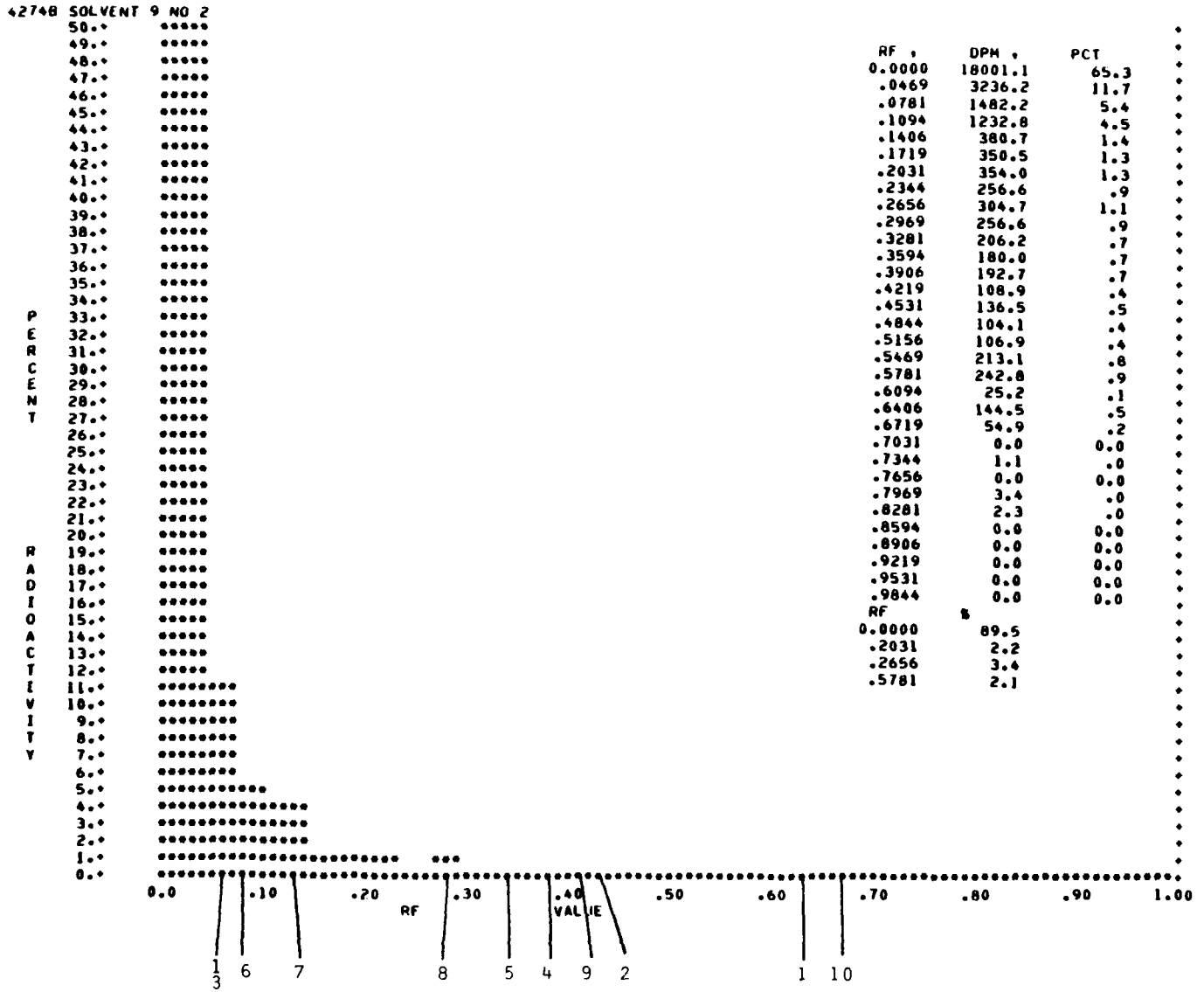

Figure 14-d-IX: Female Rats, Dermal Application, Solvent IX

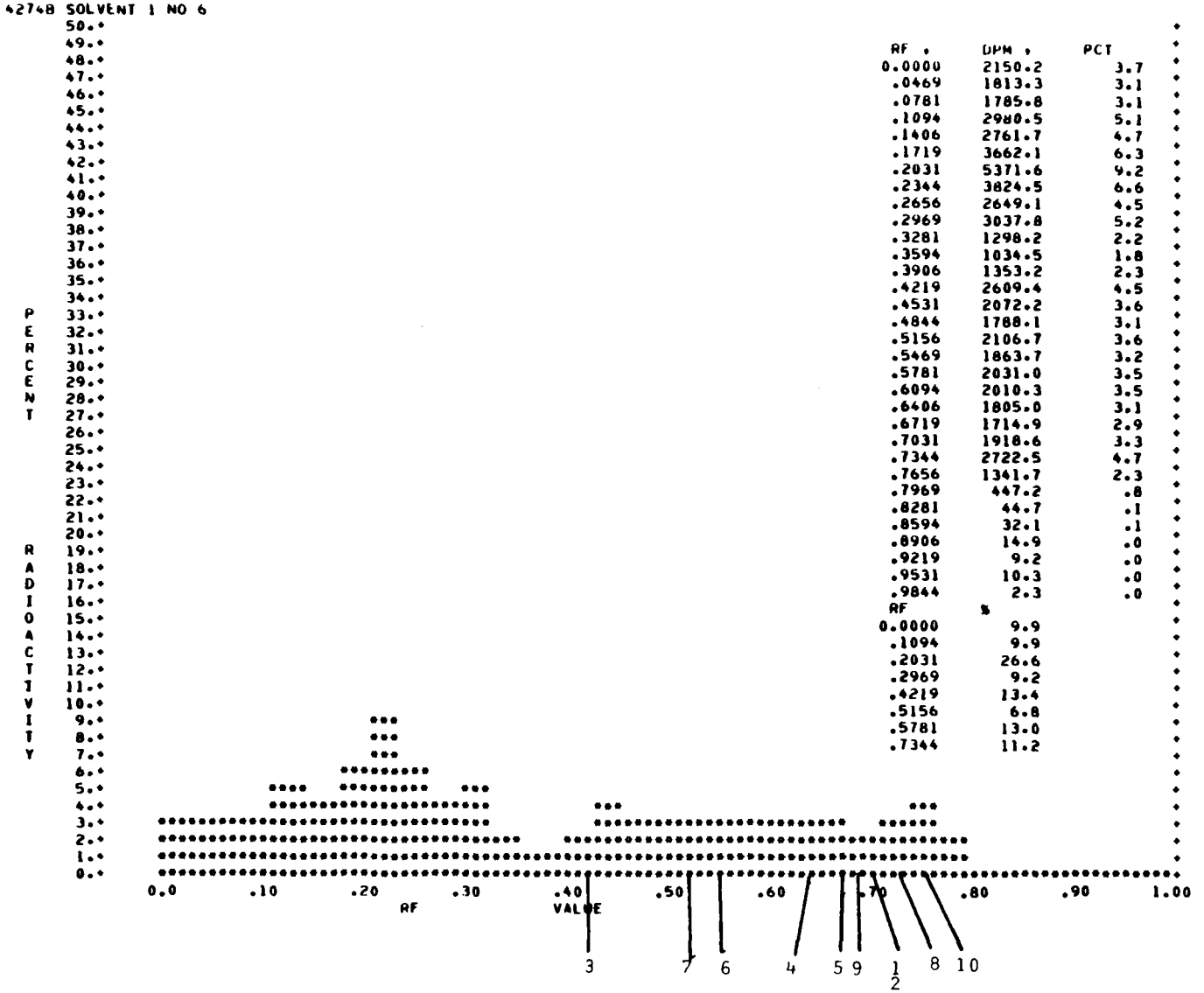

Figure 14-e-I: Male Mice, Oral Treatment, Solvent I

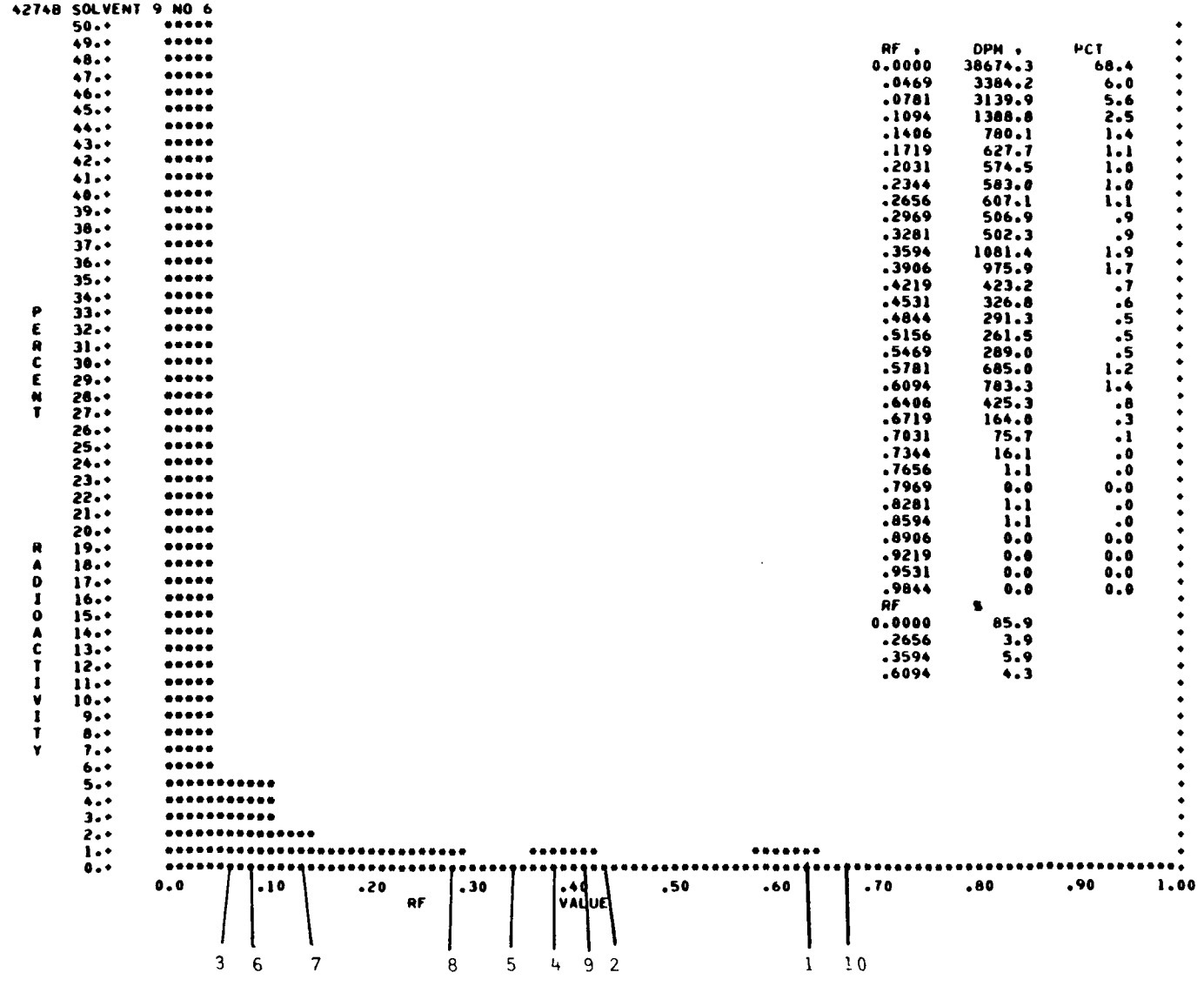

Figure 14-e-IX: Male Mice, Oral Treatment, Solvent IX

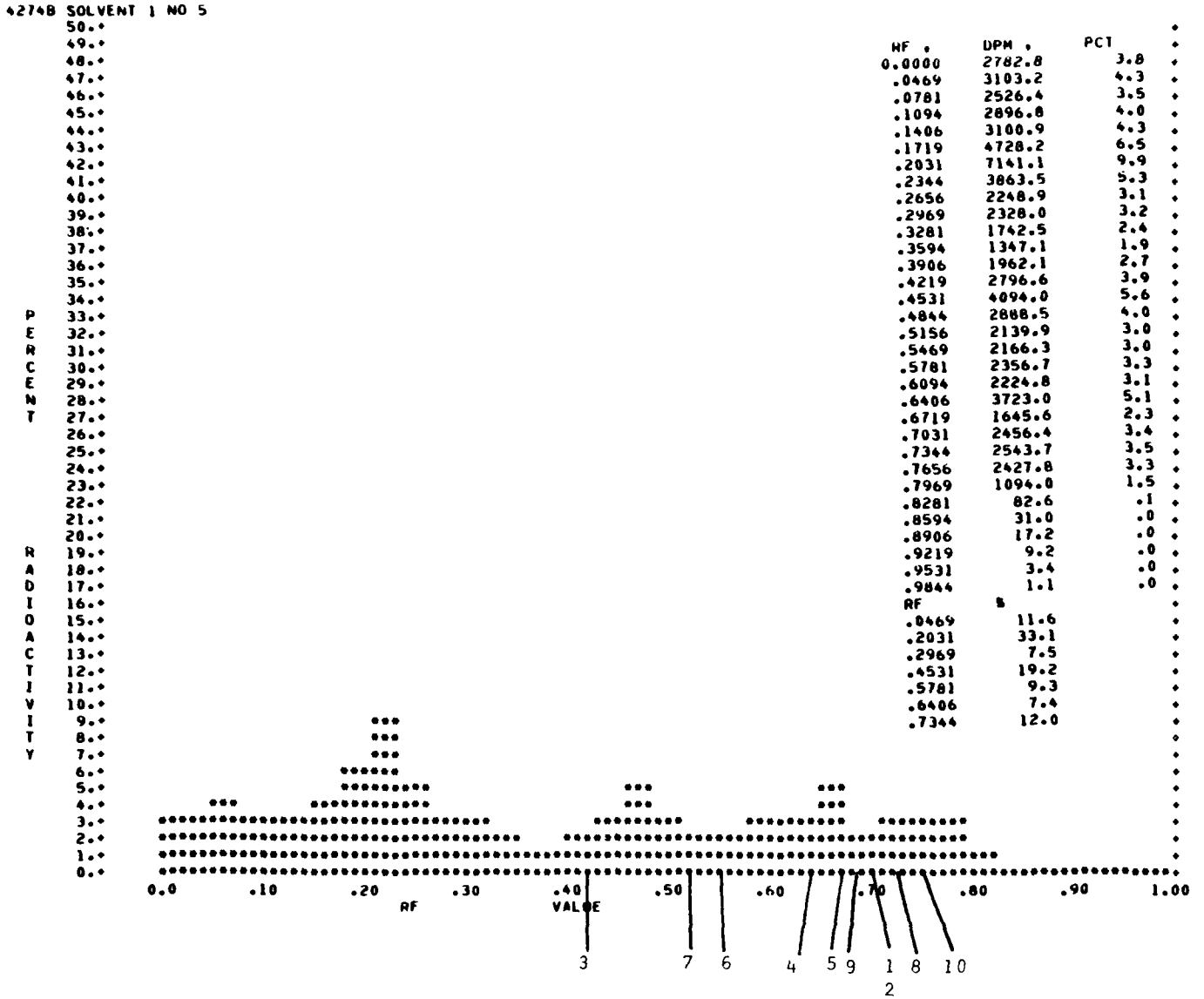

Figure 14-f-I: Male Mice, Dermal Application, Solvent I

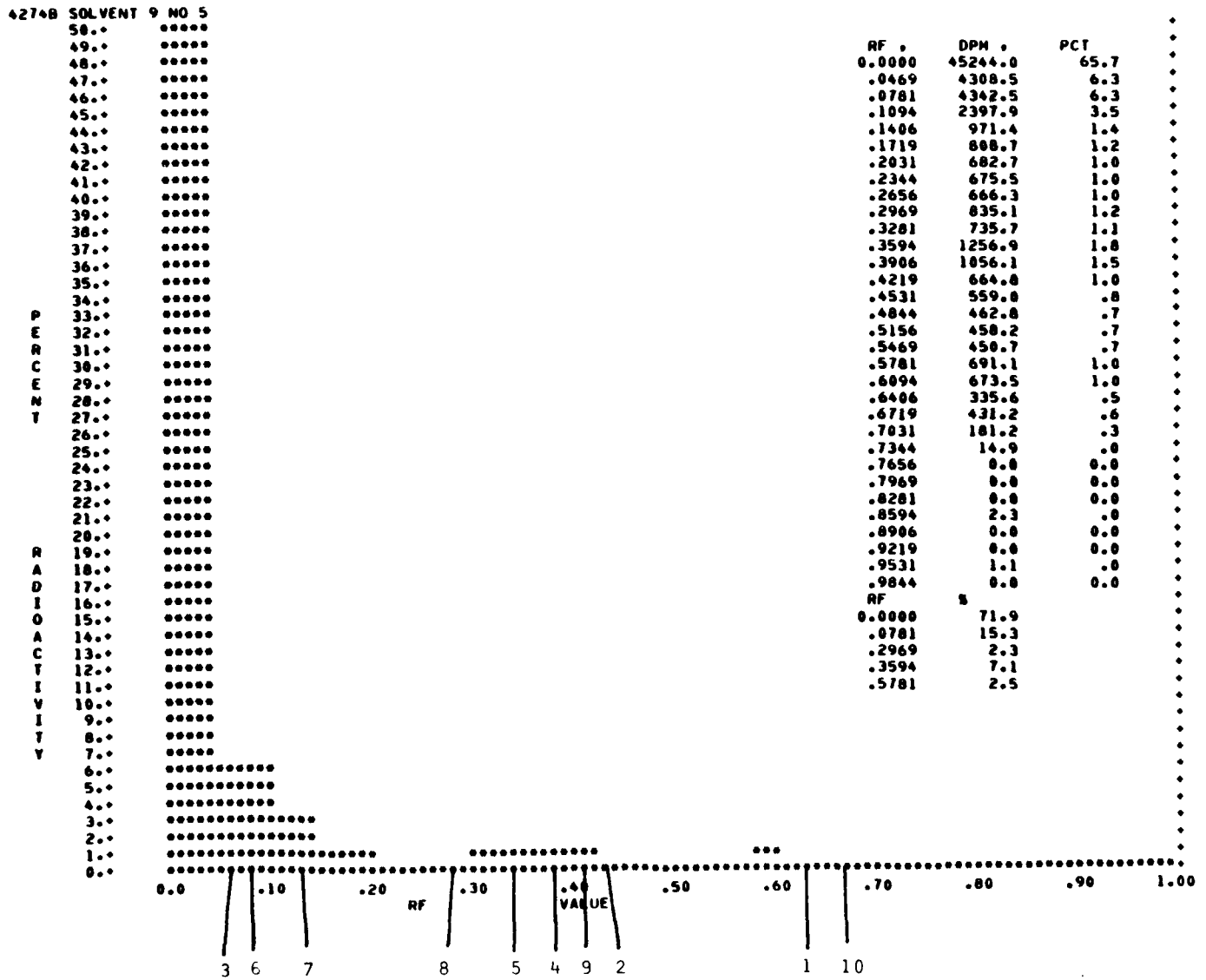

Figure 14-f-IX: Male Mice, Dermal Application, Solvent IX

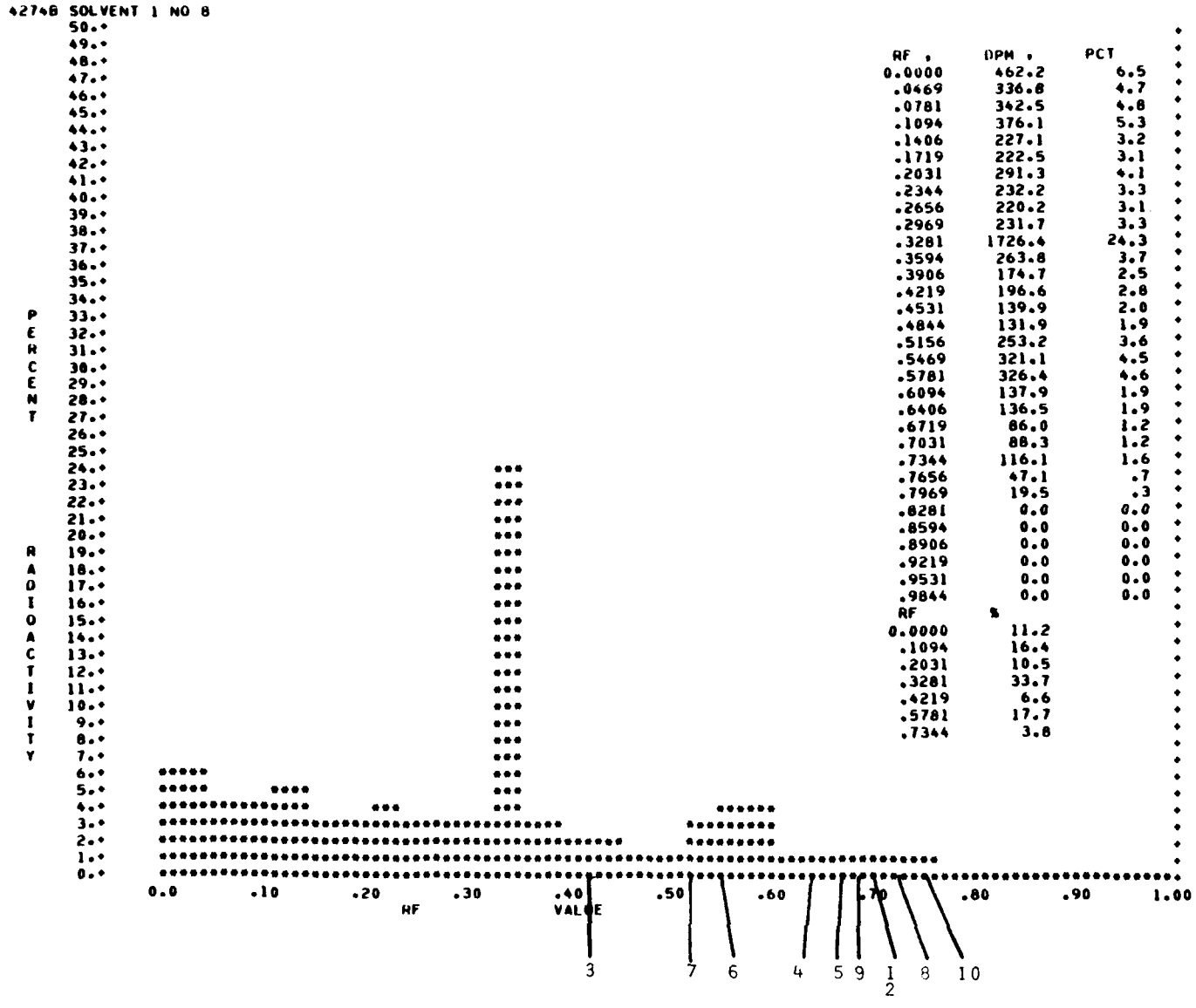

Figure 14-g-I: Male Rabbits, Oral Treatment, Solvent I

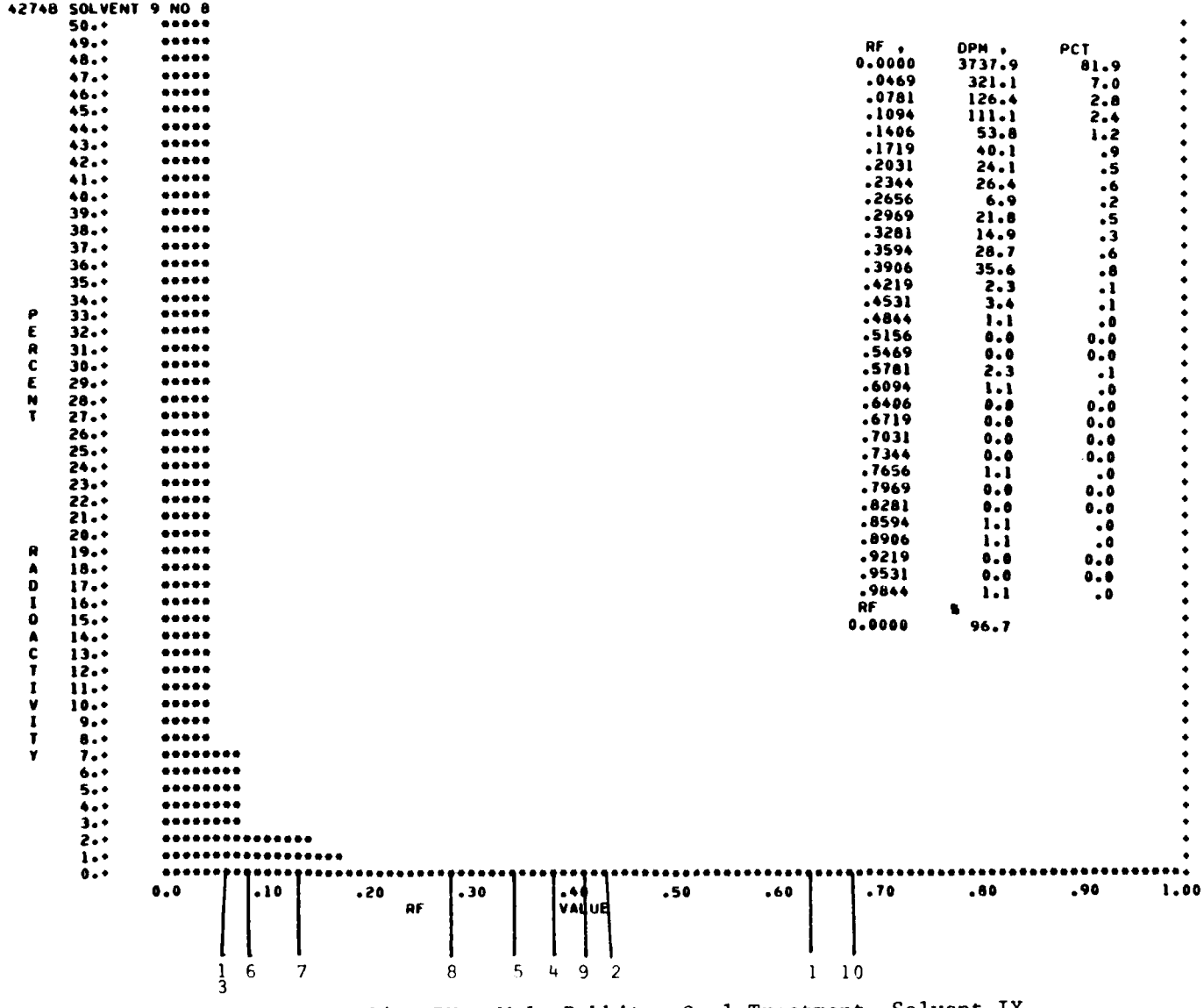

Figure 14-g-IX: Male Rabbits, Oral Treatment, Solvent IX

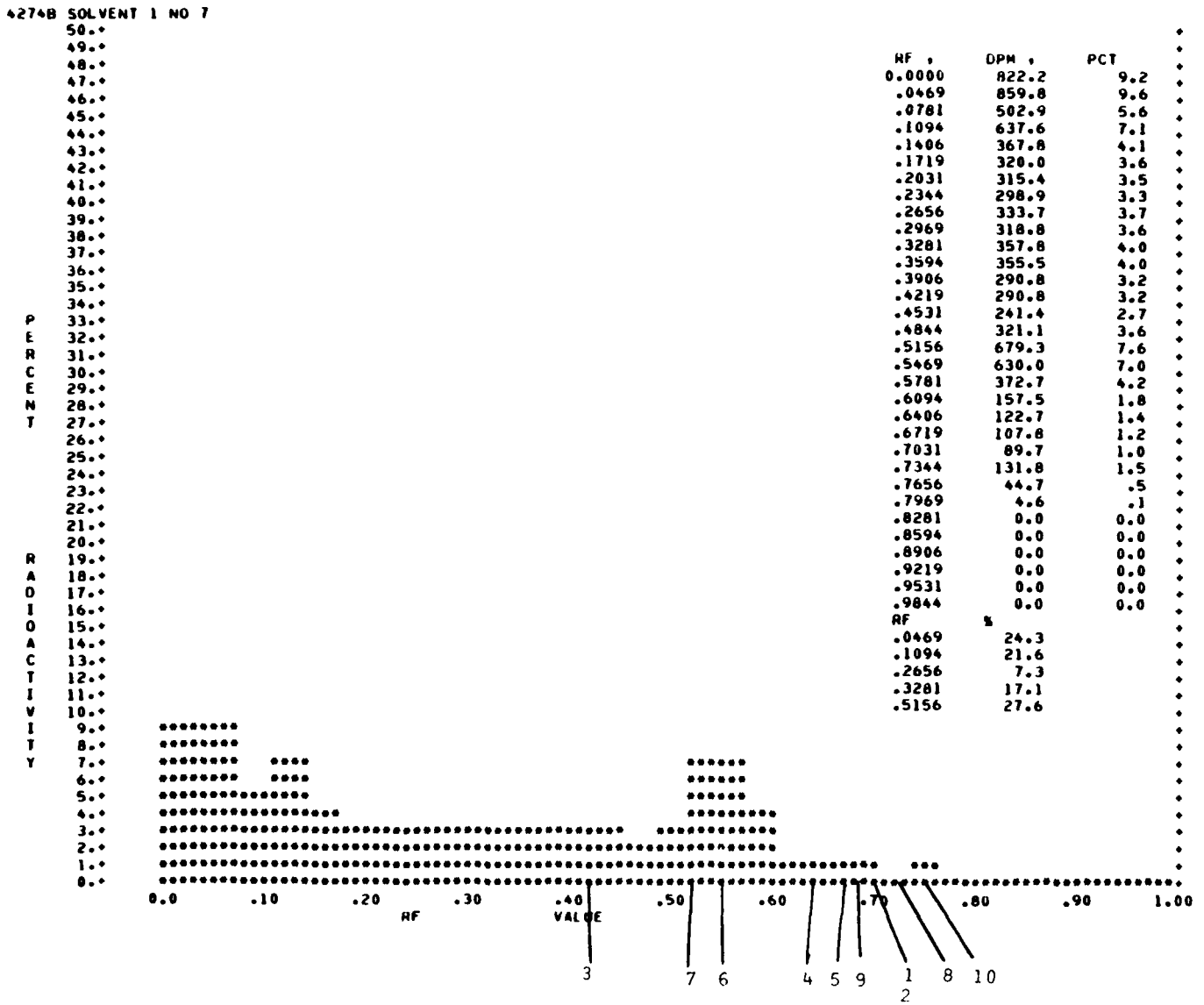

Figure 14-h-I: Male Rabbits, Dermal Application, Solvent I

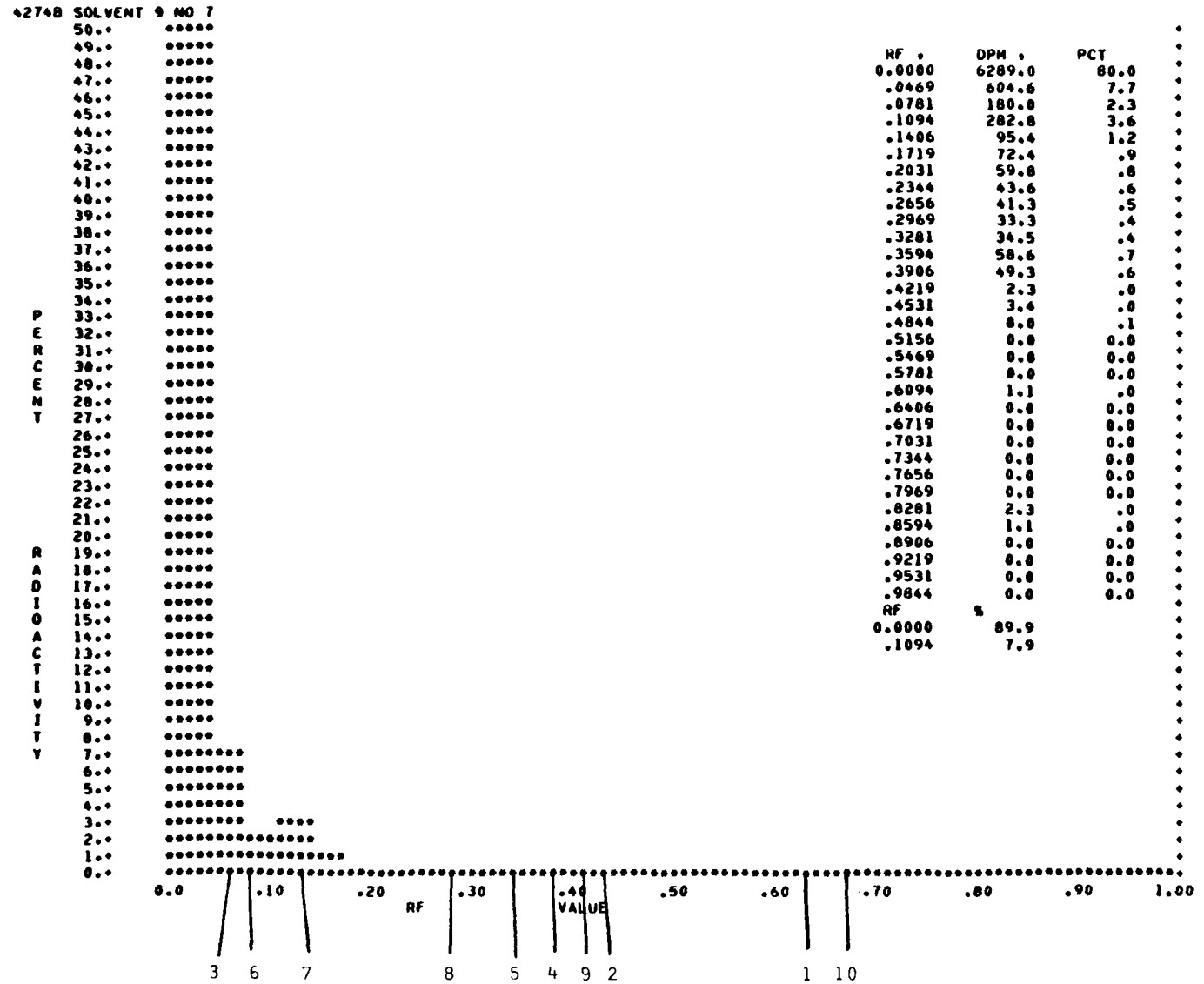

Figure 14-h-IX: Male Rabbits, Dermal Application, Solvent IX

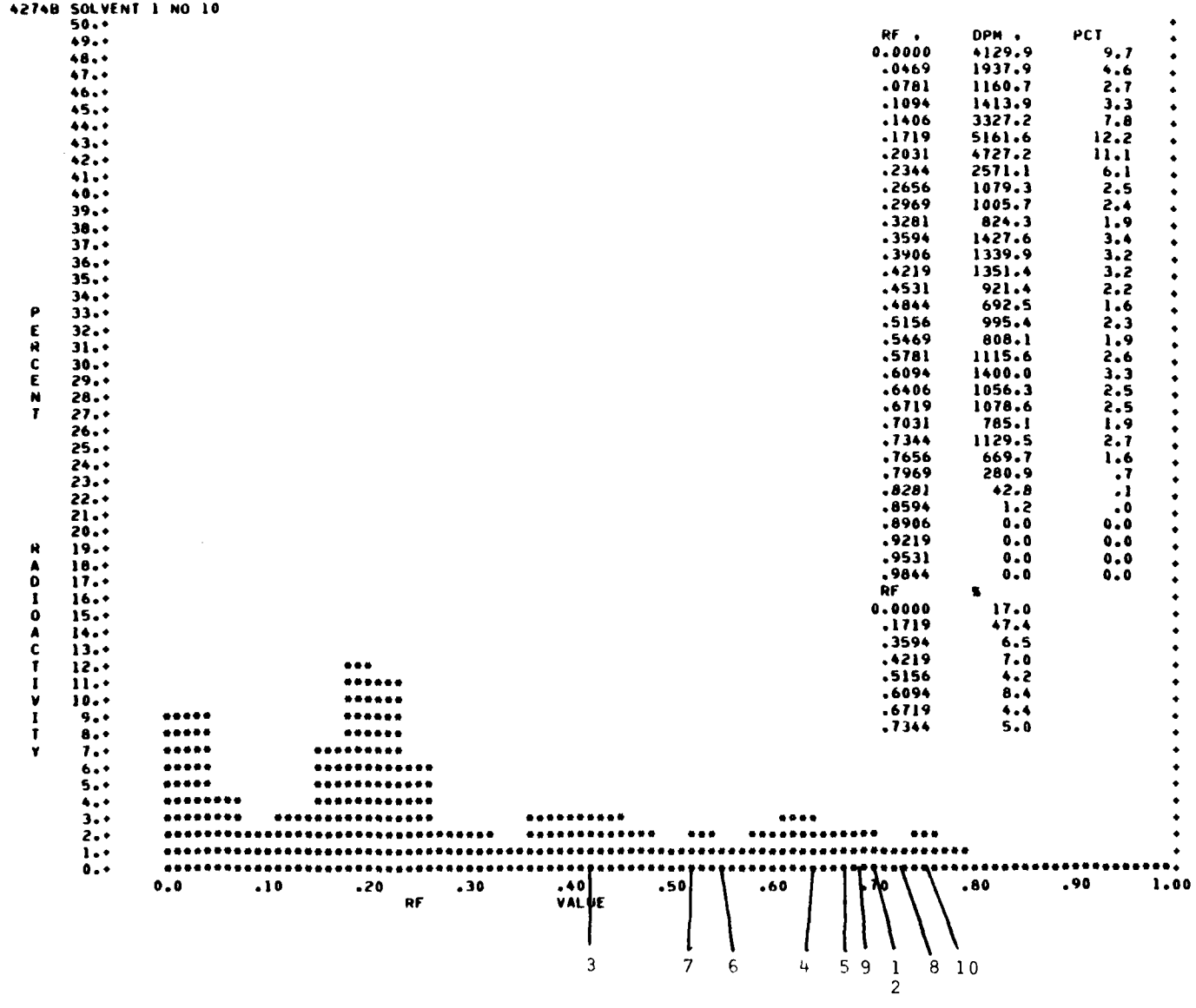

Figure 14-k-I: Male Dogs, Oral Treatment, Solvent I

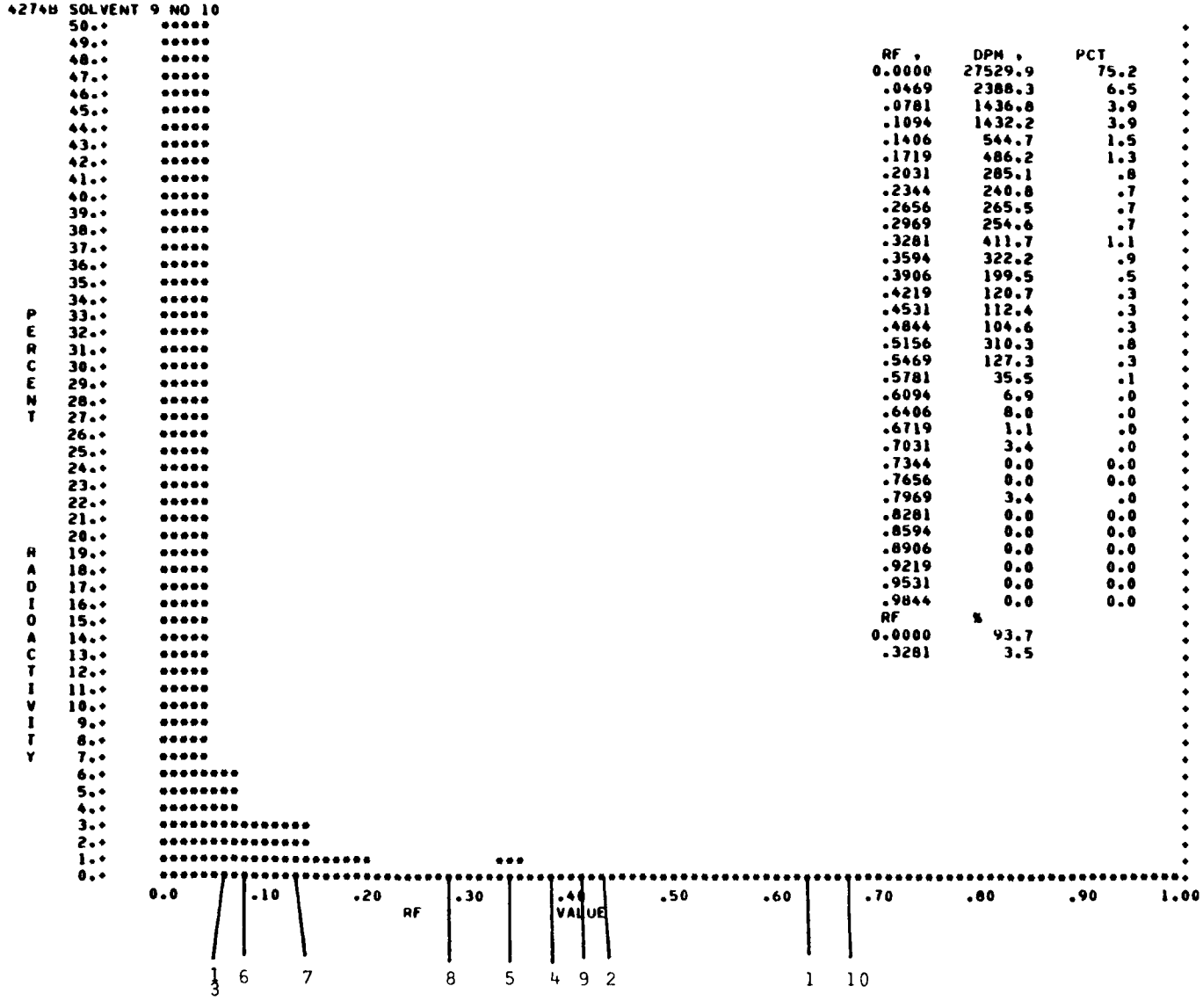

Figure 14-k-IX: Male Dogs, Oral Treatment, Solvent IX

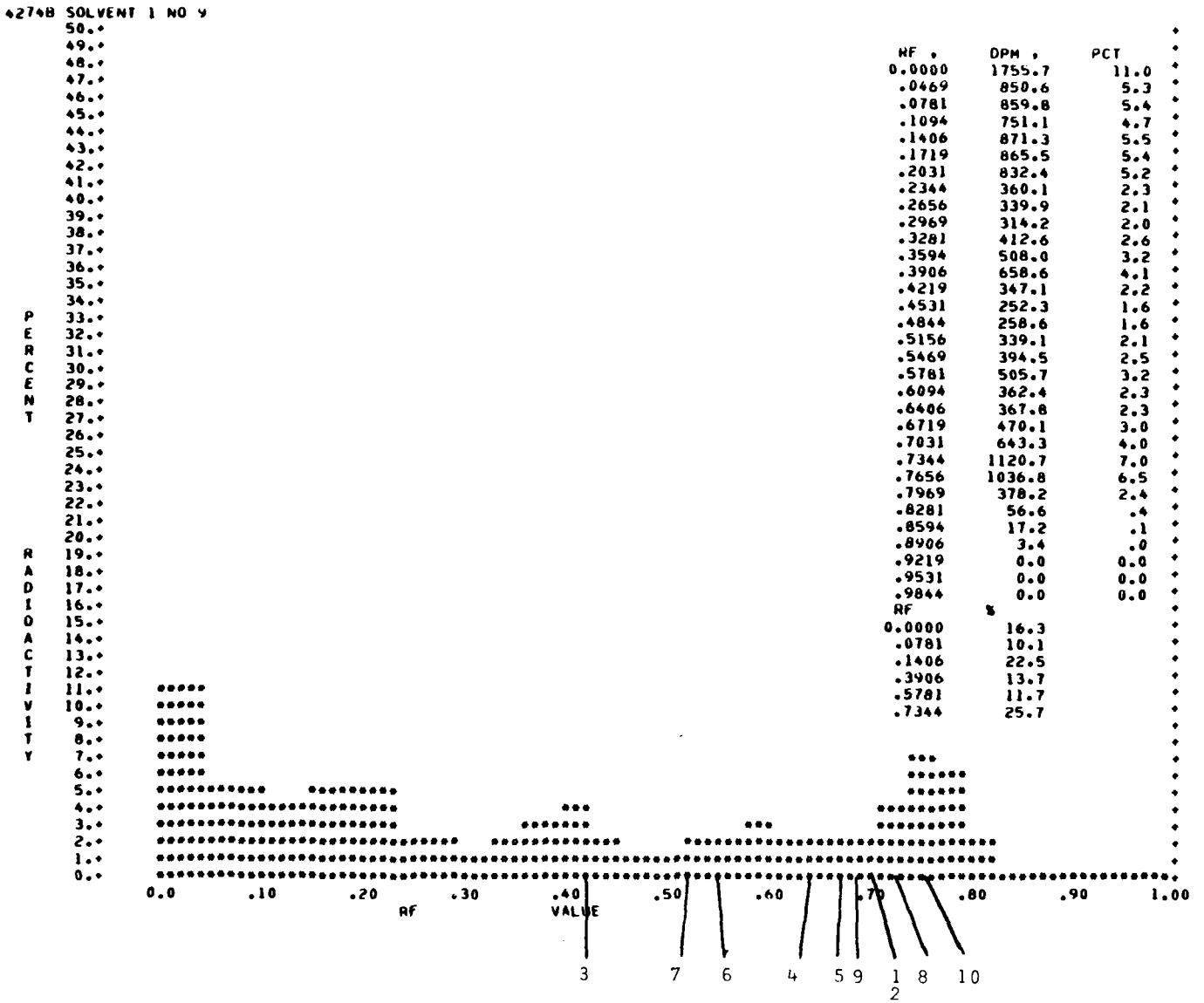

Figure 14-1-I: Male Dogs, Dermal Application, Solvent I

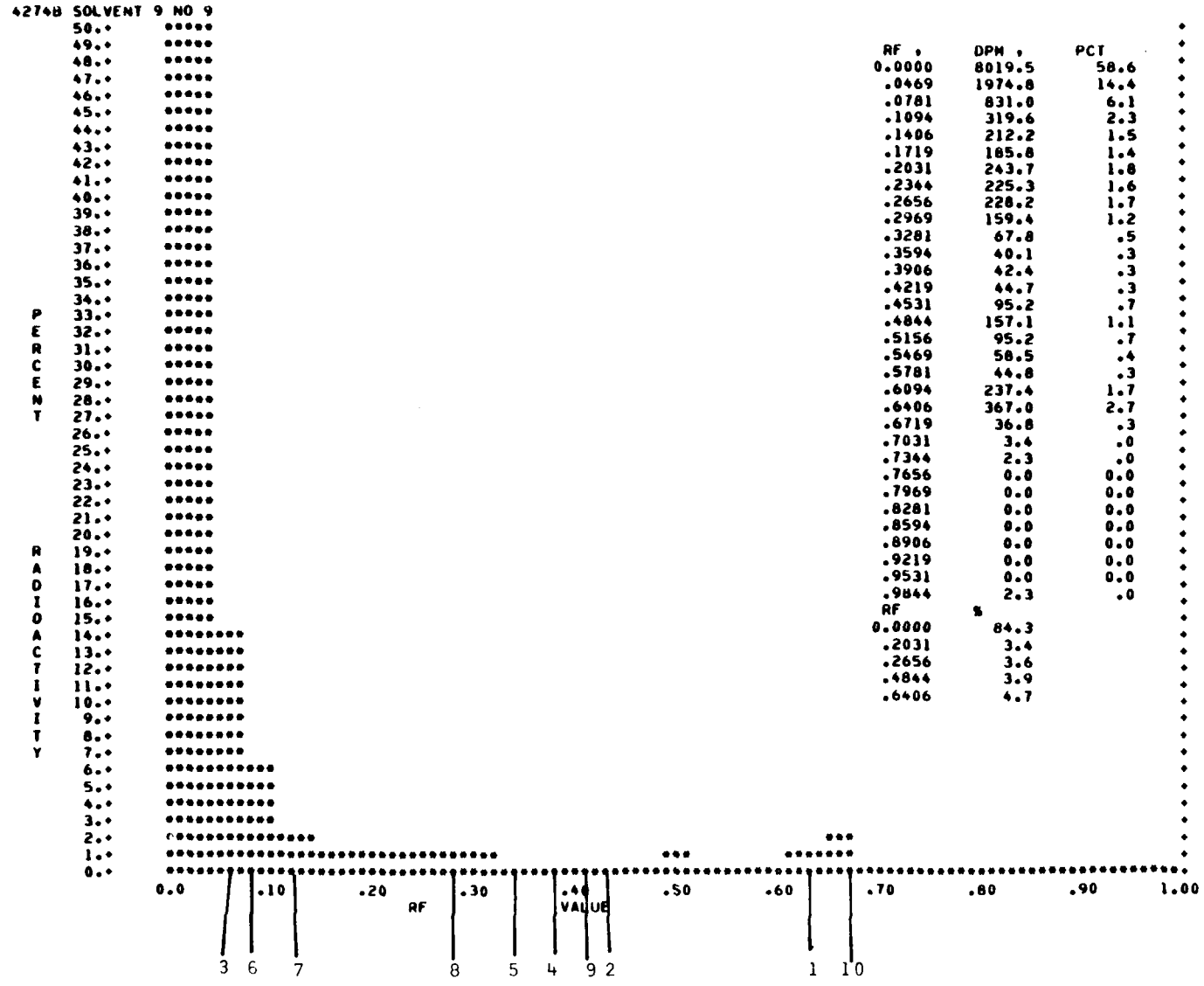

Figure 14-1-IX: Male Dogs, Dermal Application, Solvent IX

Figure 15: TLC of Ethyl Acetate-Extractable Products Obtained from 24-Hr Urine of Rats Treated Orally with ^{14}C-TNT. Prior to extraction, urine samples were incubated with acetate buffer and β-glucuronidase or aryl-sulfatase control samples were incubated with acetate buffer and water. Reference standards are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzylalcohol
3. Trinitrobenzoic Acid
4. 4-Amino-2,6-Dinitrotoluene
5. 2-Amino-4,6-Dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2,6'-Tetranitro-4,4'-azoxytoluene

Figure 15 follows

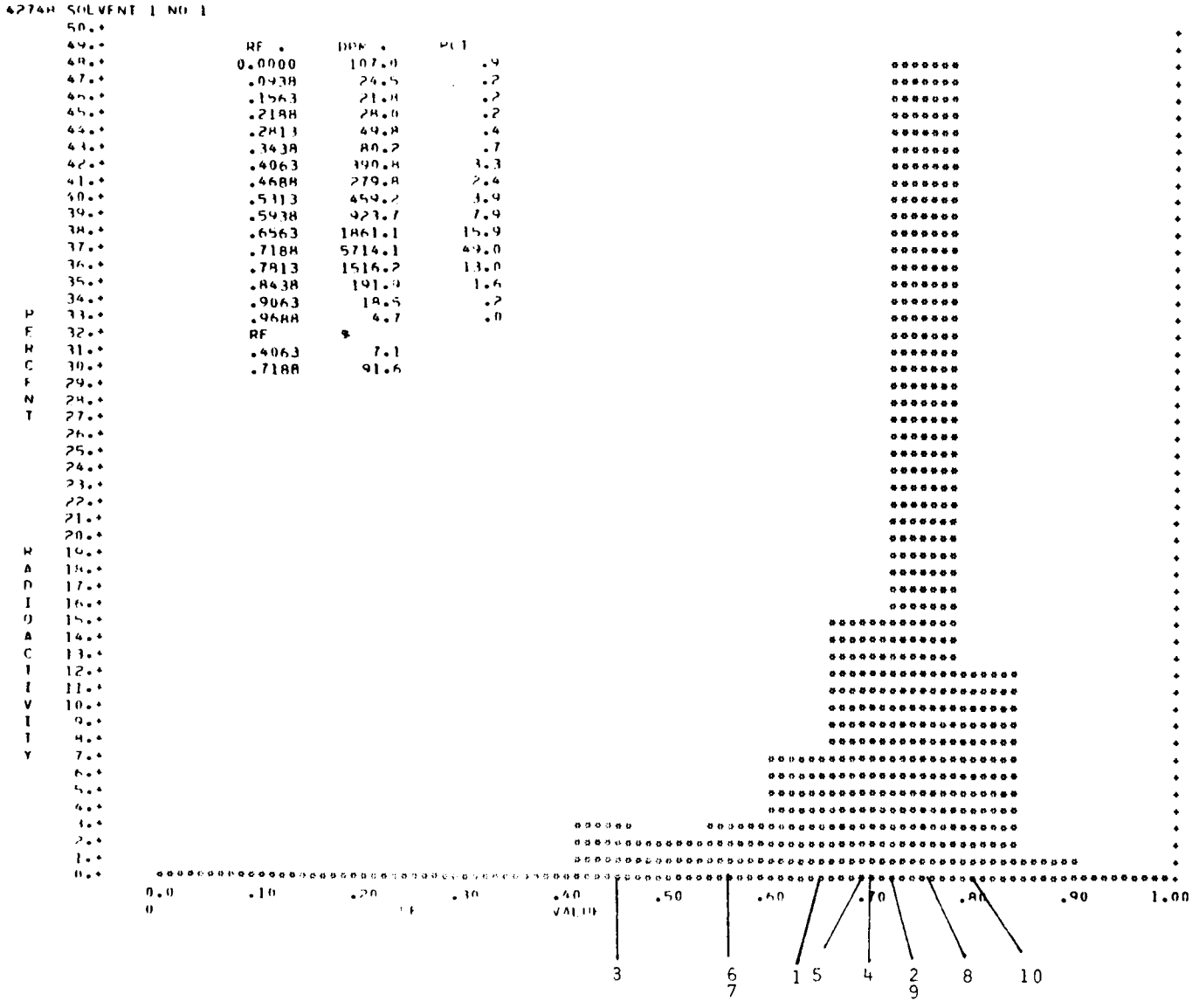

Figure 15-a-I: Male Rats, Incubation with Water, Solvent I

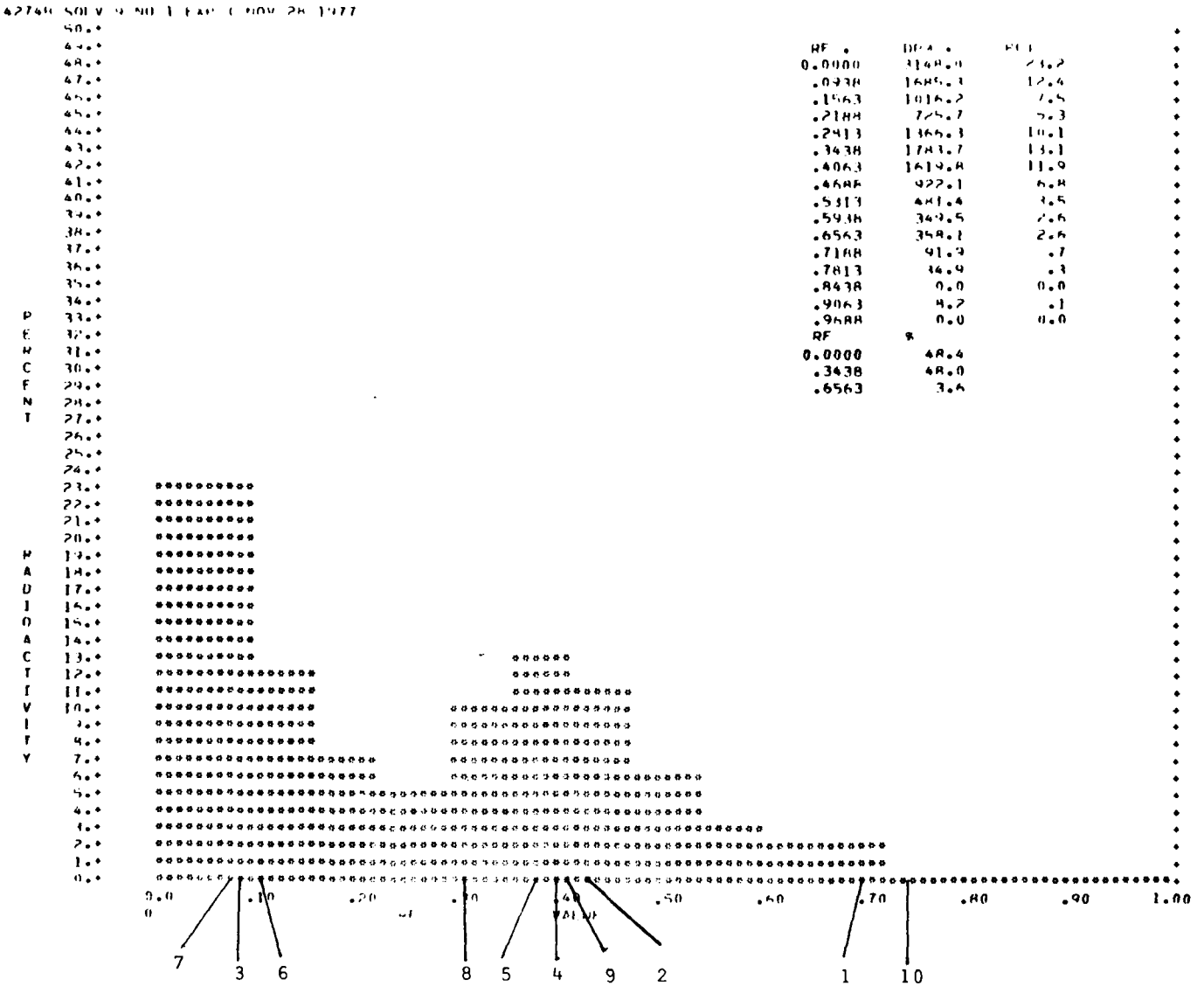

Figure 15-a-IX: Male Rats, Incubation with Water, Solvent IX

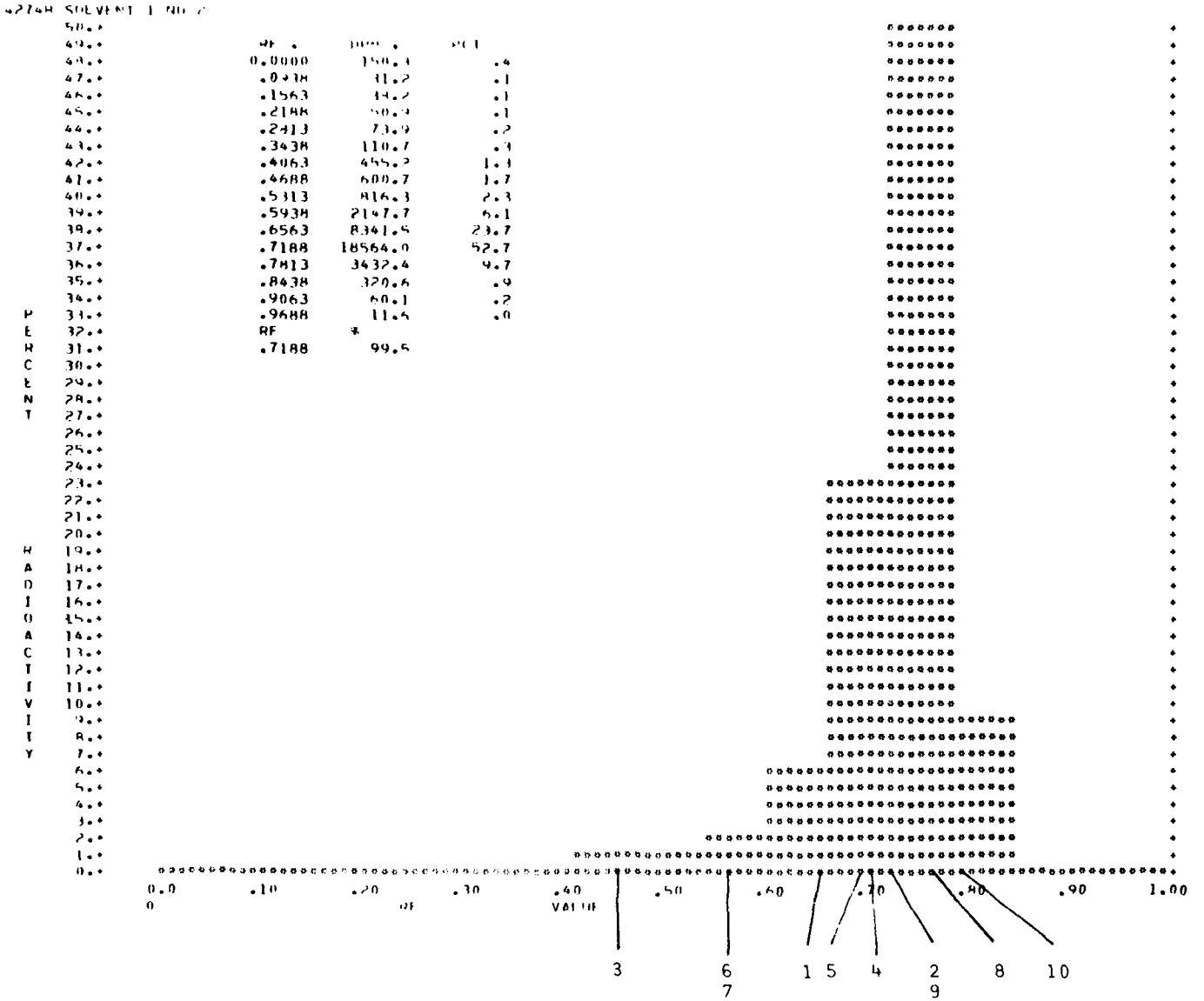

Figure 15-b-I: Male Rats, Incubation with β-Glucuronidase, Solvent I

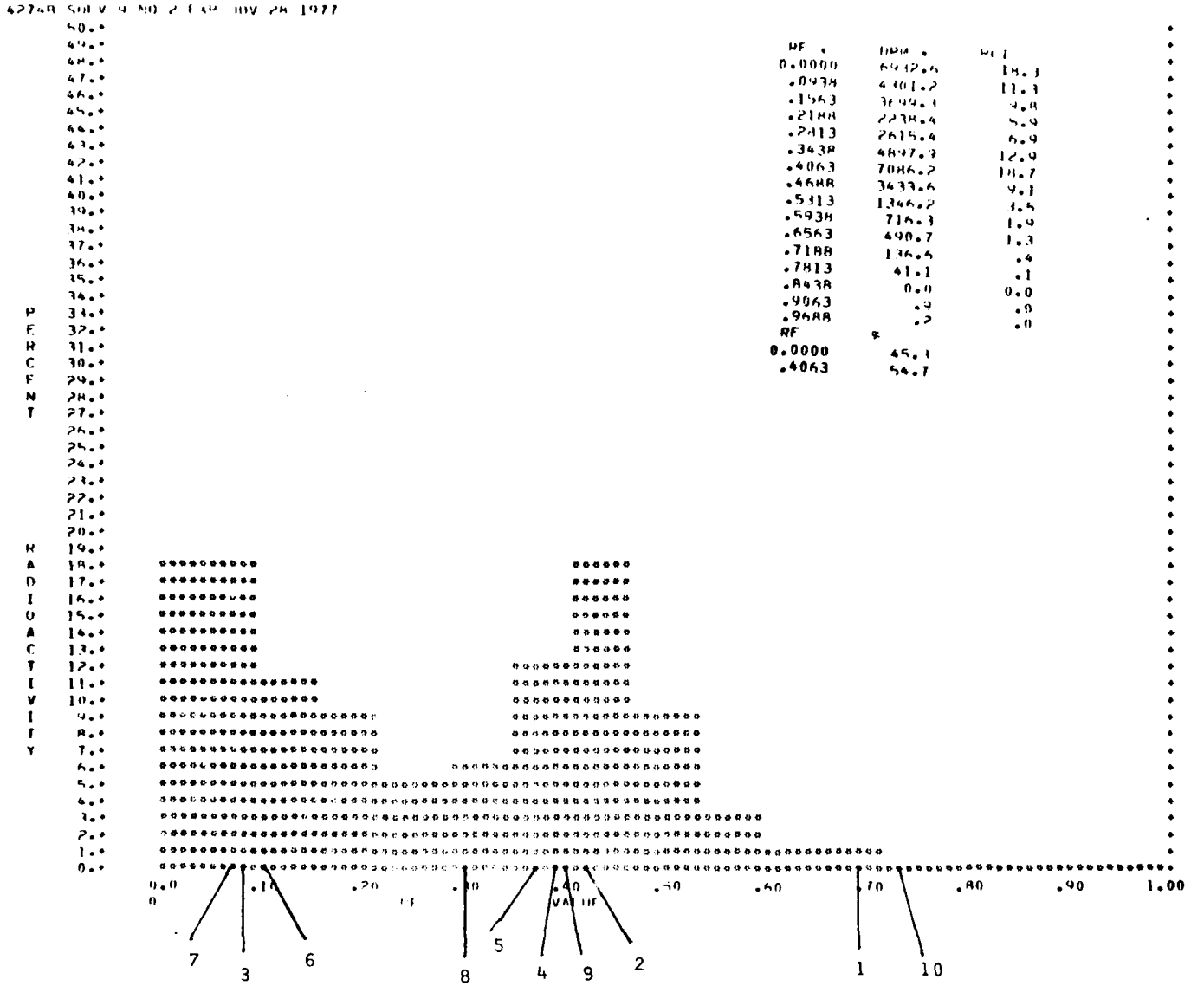

Figure 15-b-IX: Male Rats, Incubation with β-Glucuronidase, Solvent IX

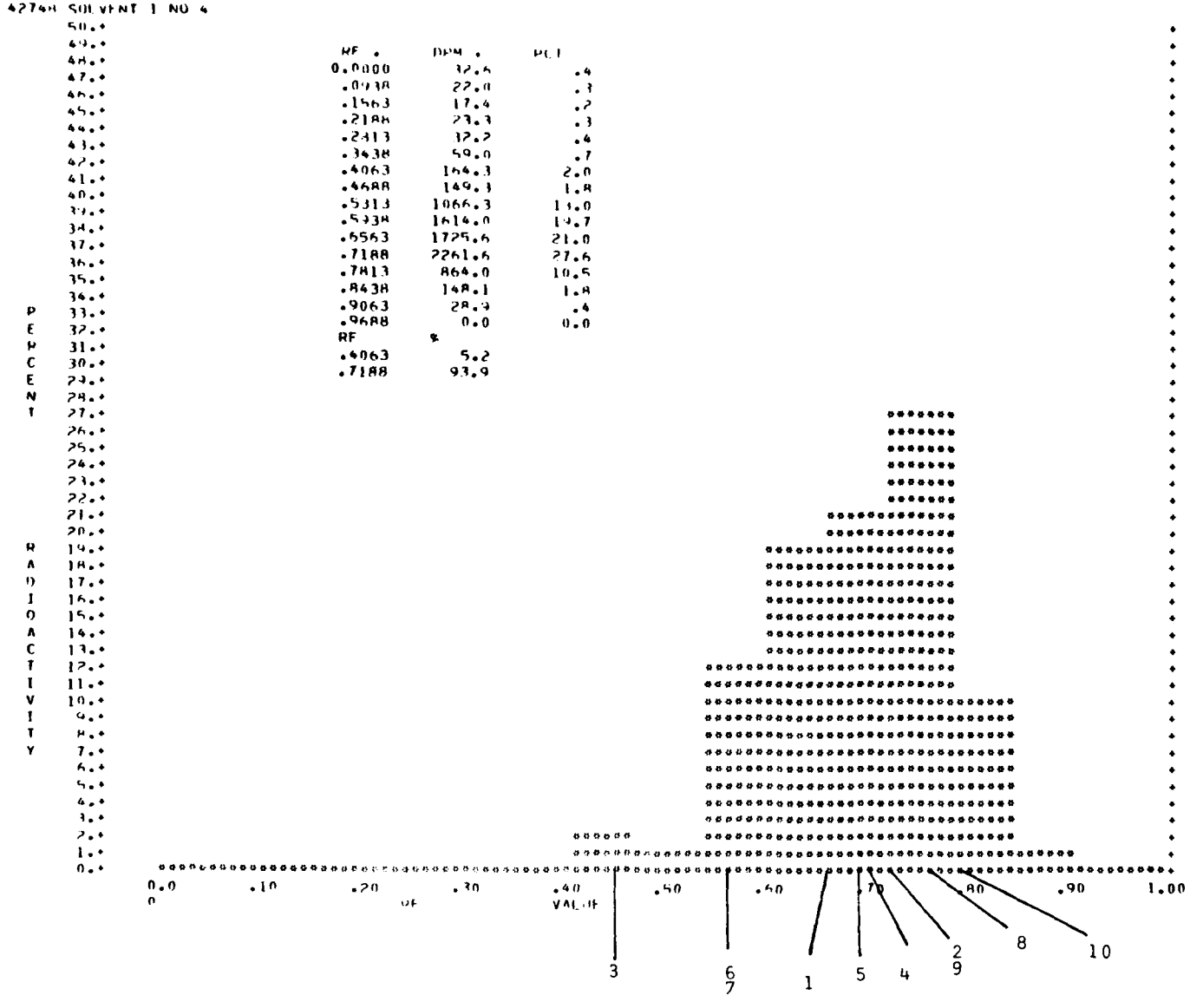

Figure 15-c-I: Male Rats, Incubation with Aryl Sulfatase, Solvent I

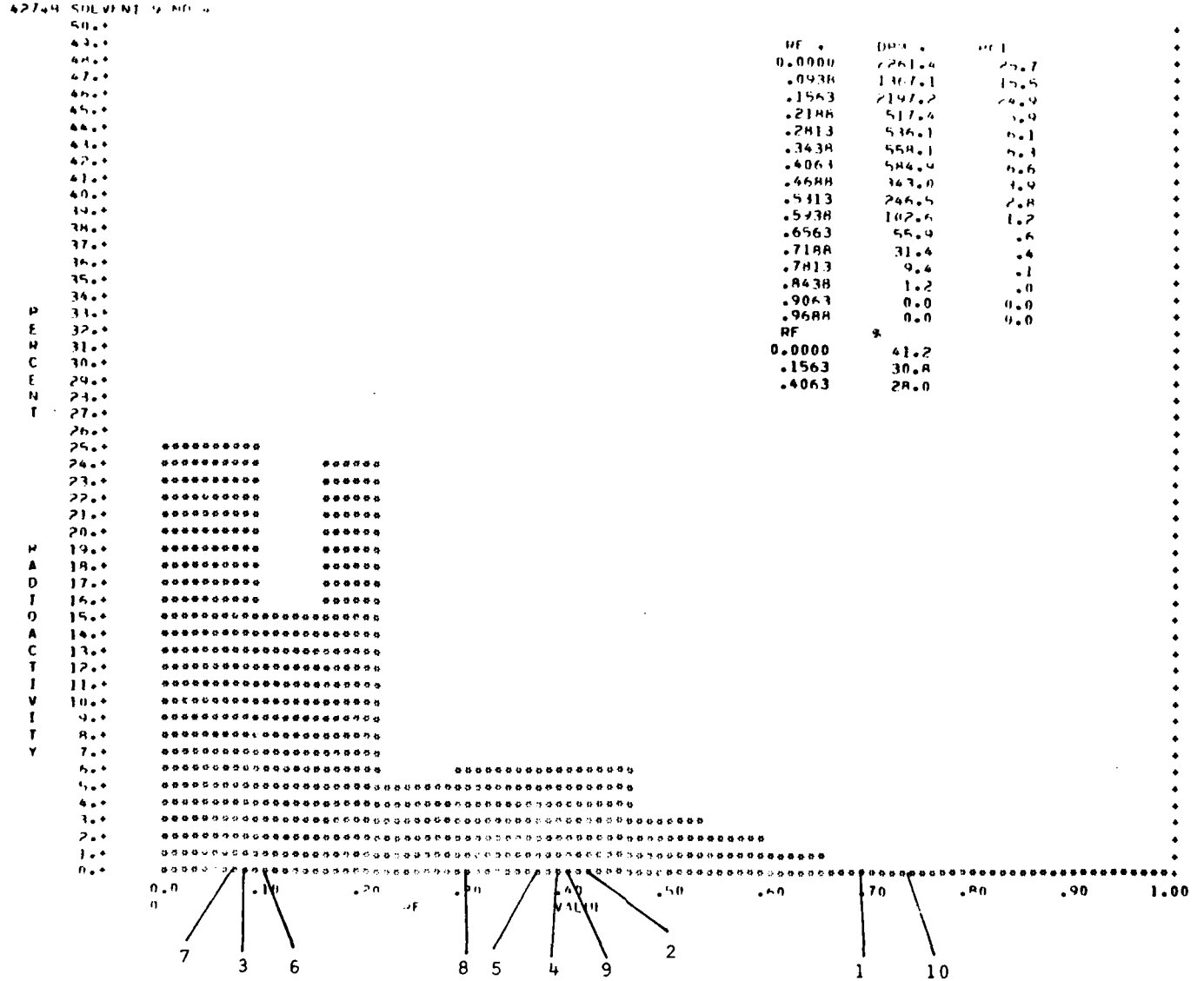

Figure 15-c-IX: Male Rats, Incubation with Aryl Sulfatase, Solvent IX

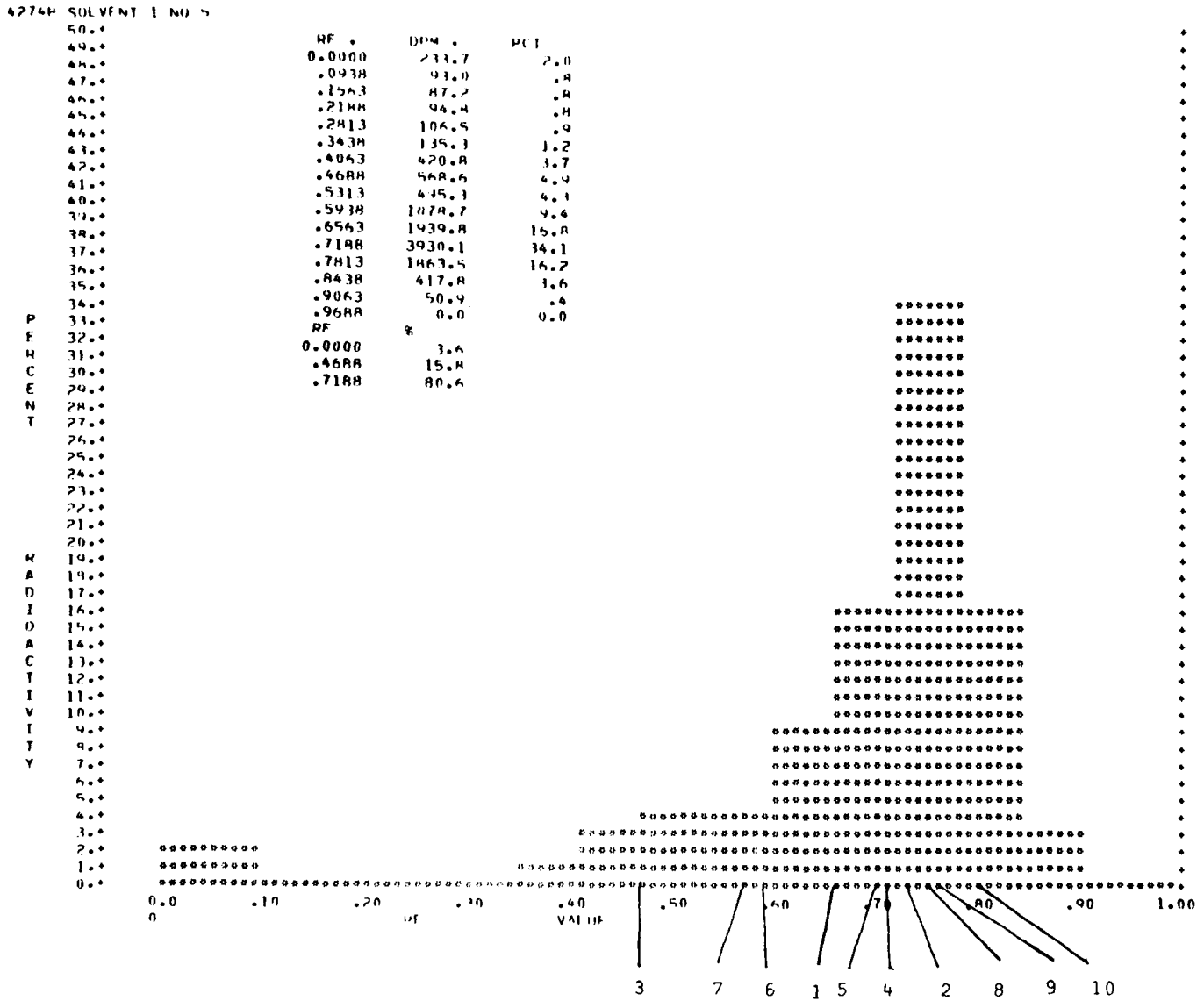

Figure 15-d-I: Female Rats, Incubation with Water, Solvent I

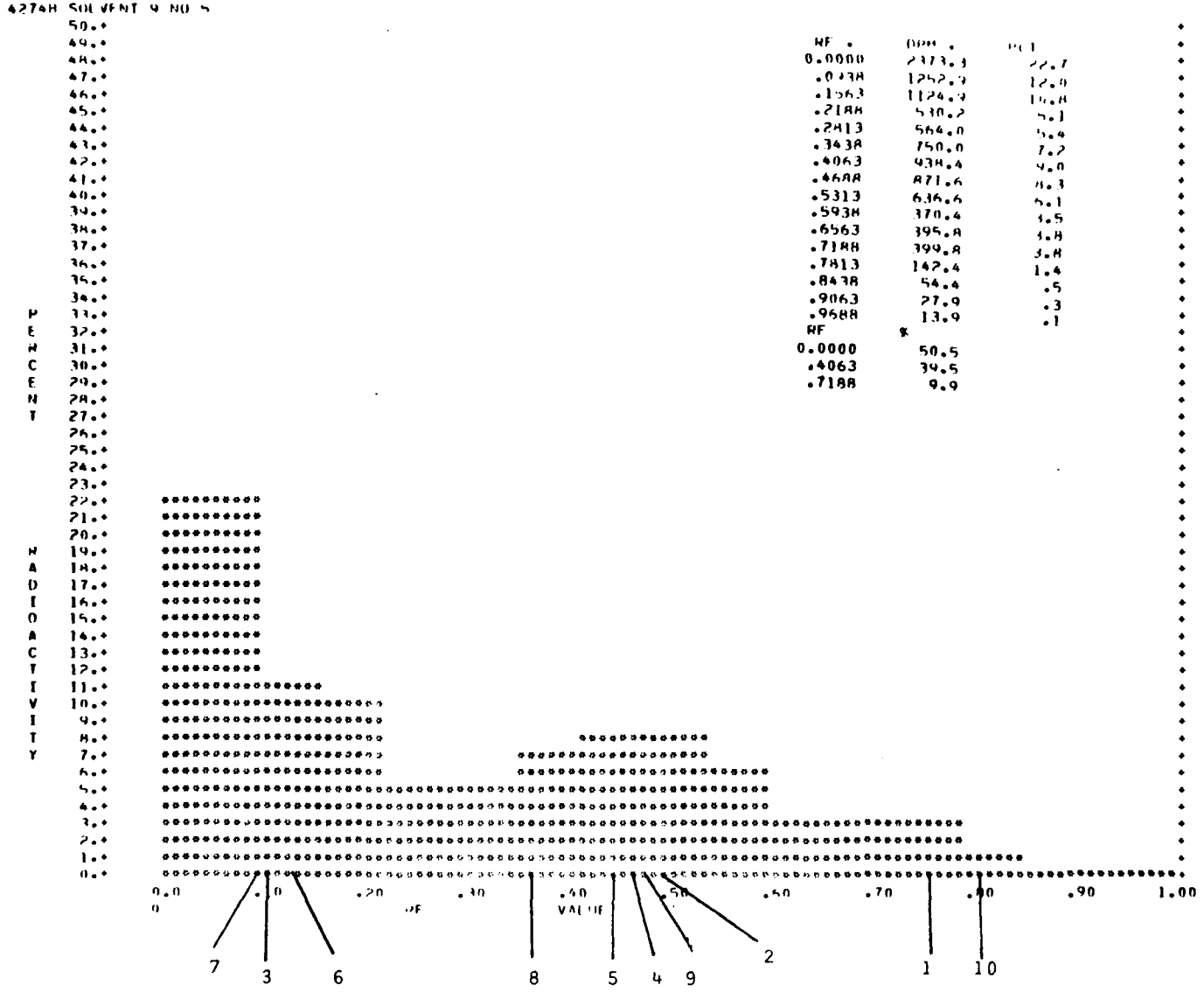

Figure 15-d-IX: Female Rats, Incubation with Water, Solvent IX

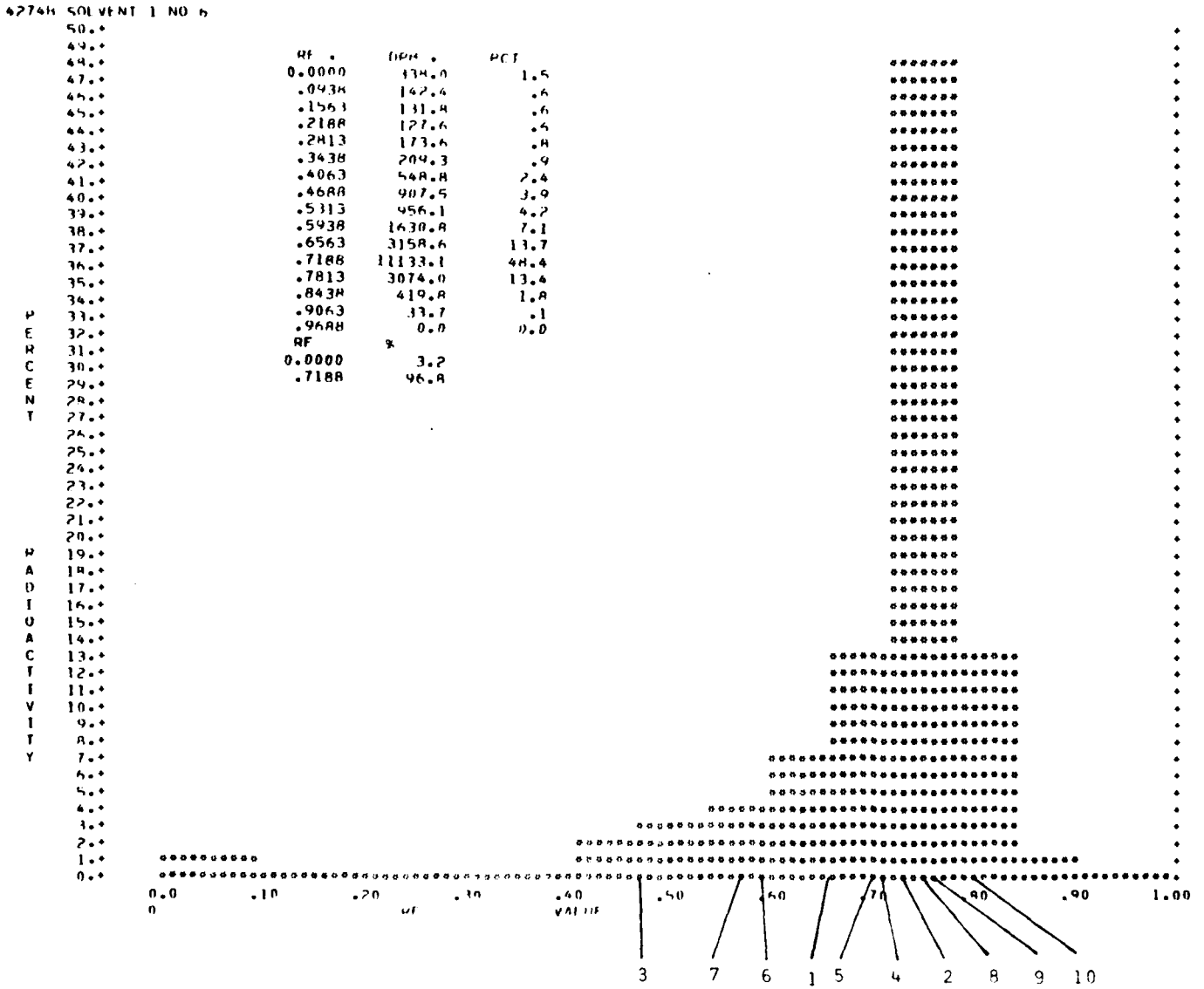

Figure 15-e-I: Female Rats, Incubation with β-Glucuronidase, Solvent I

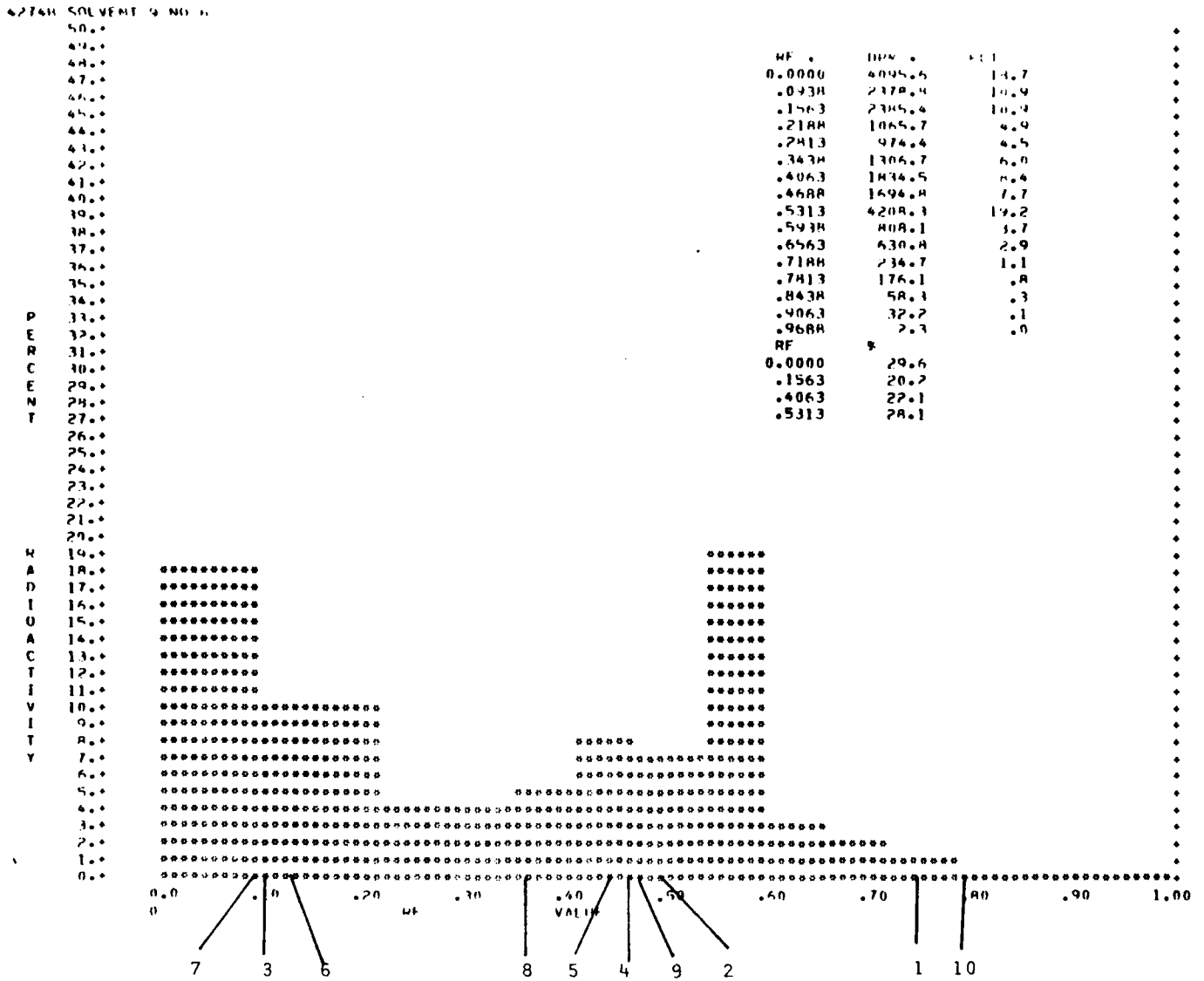

Figure 15-e-IX: Female Rats, Incubation with β-Glucuronidase, Solvent IX

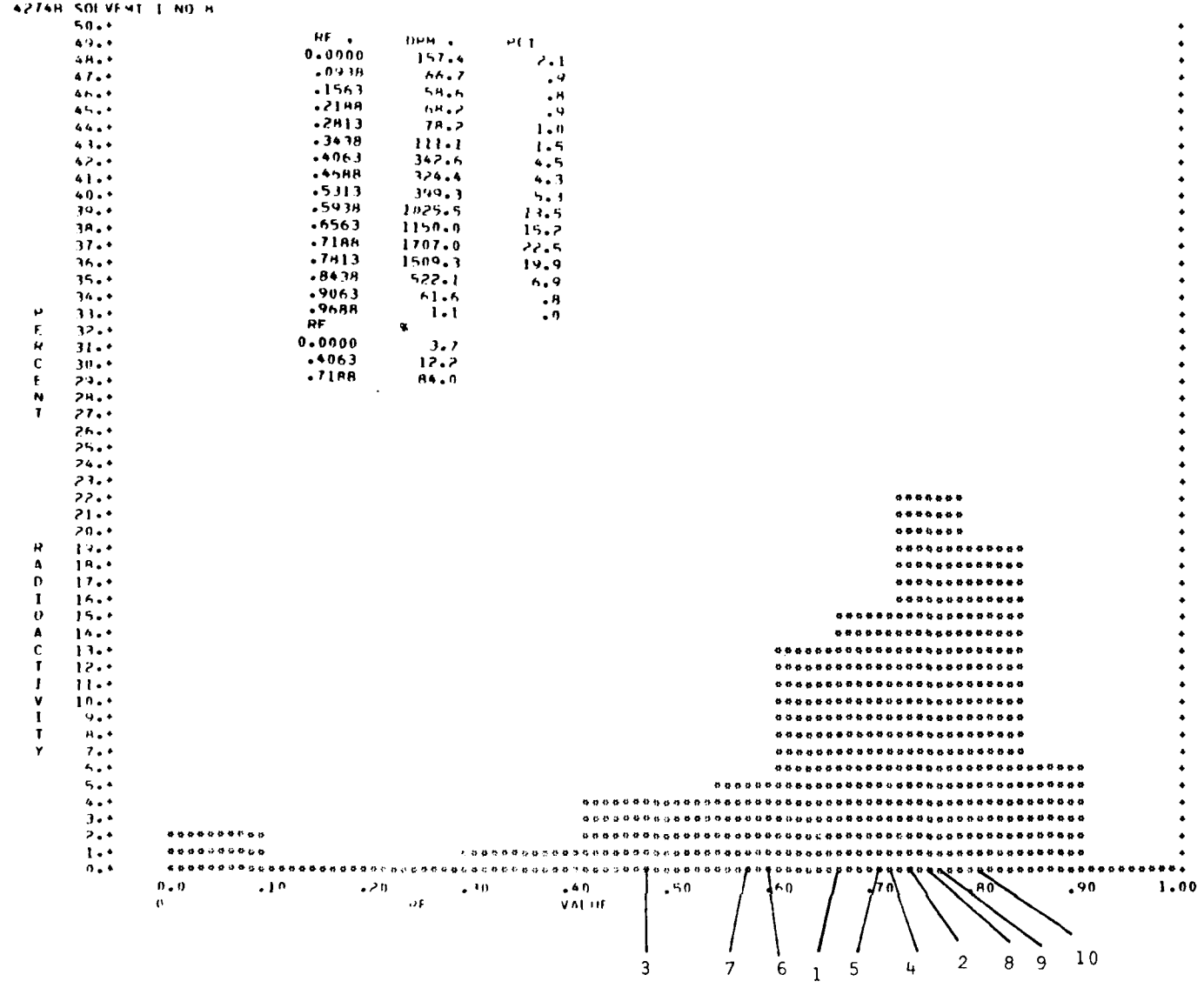

Figure 15-f-I: Female Rats, Incubation with Aryl Sulfatase, Solvent I

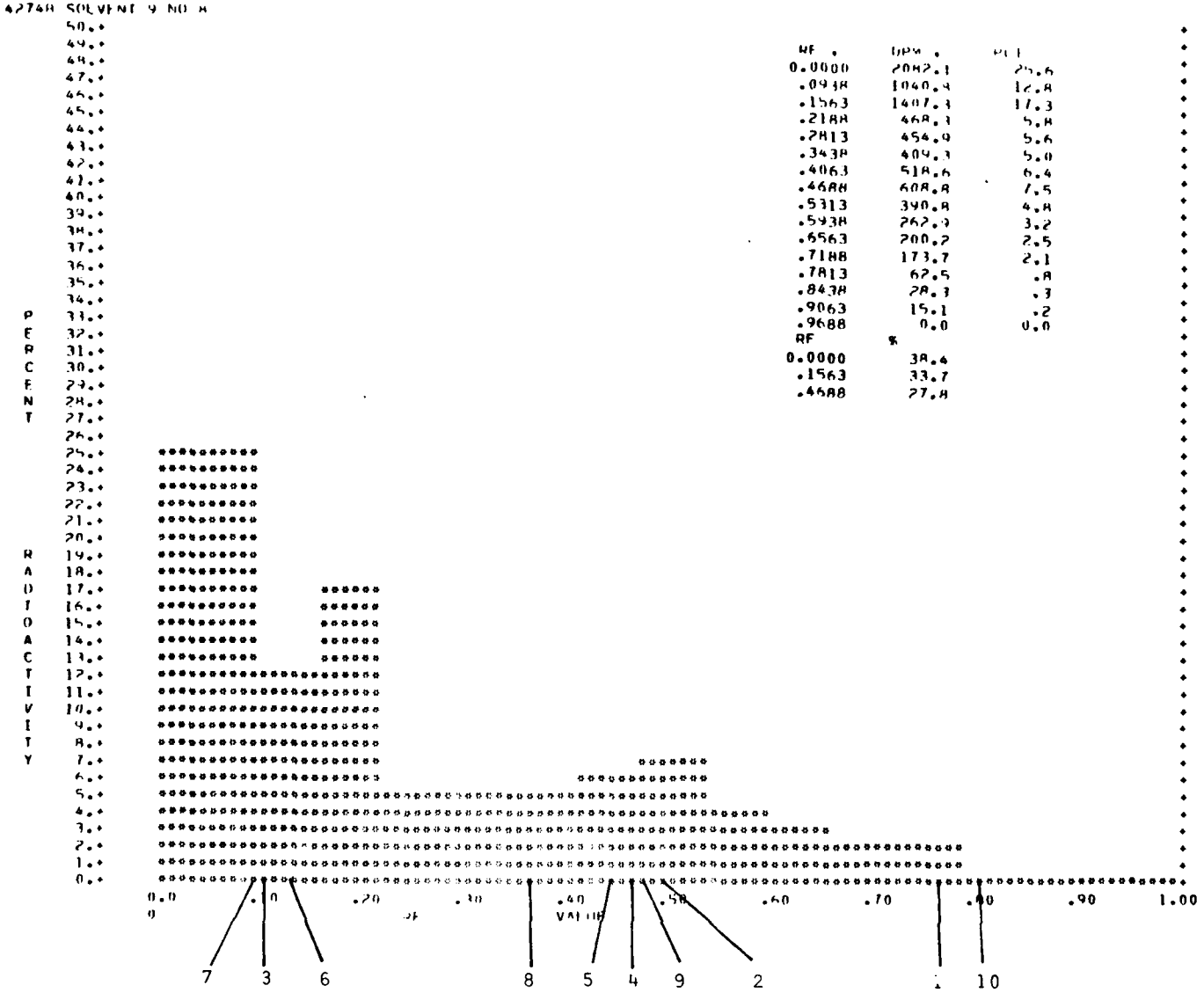

Figure 15-f-IX: Female Rats, Incubation with Aryl Sulfatase, Solvent IX

Figure 16: TLC of Ethyl Acetate-Extractable Products Obtained from 24-Hr Urine of Male Rats Treated Orally or Dermally with ^{14}C-TNT. Samples of urine were incubated with acetate buffer and β-glucuronidase before extraction with ethyl acetate. Incubation with acetate buffer and water served as control. Reference standards are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzylalcohol
3. Trinitrobenzoic Acid
4. 4-Amino-2,6-Dinitrotoluene
5. 2-Amino-4,6-Dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6pdinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2,6'-Tetranitro-4,4'-azoxytoluene

Figure 16 follows

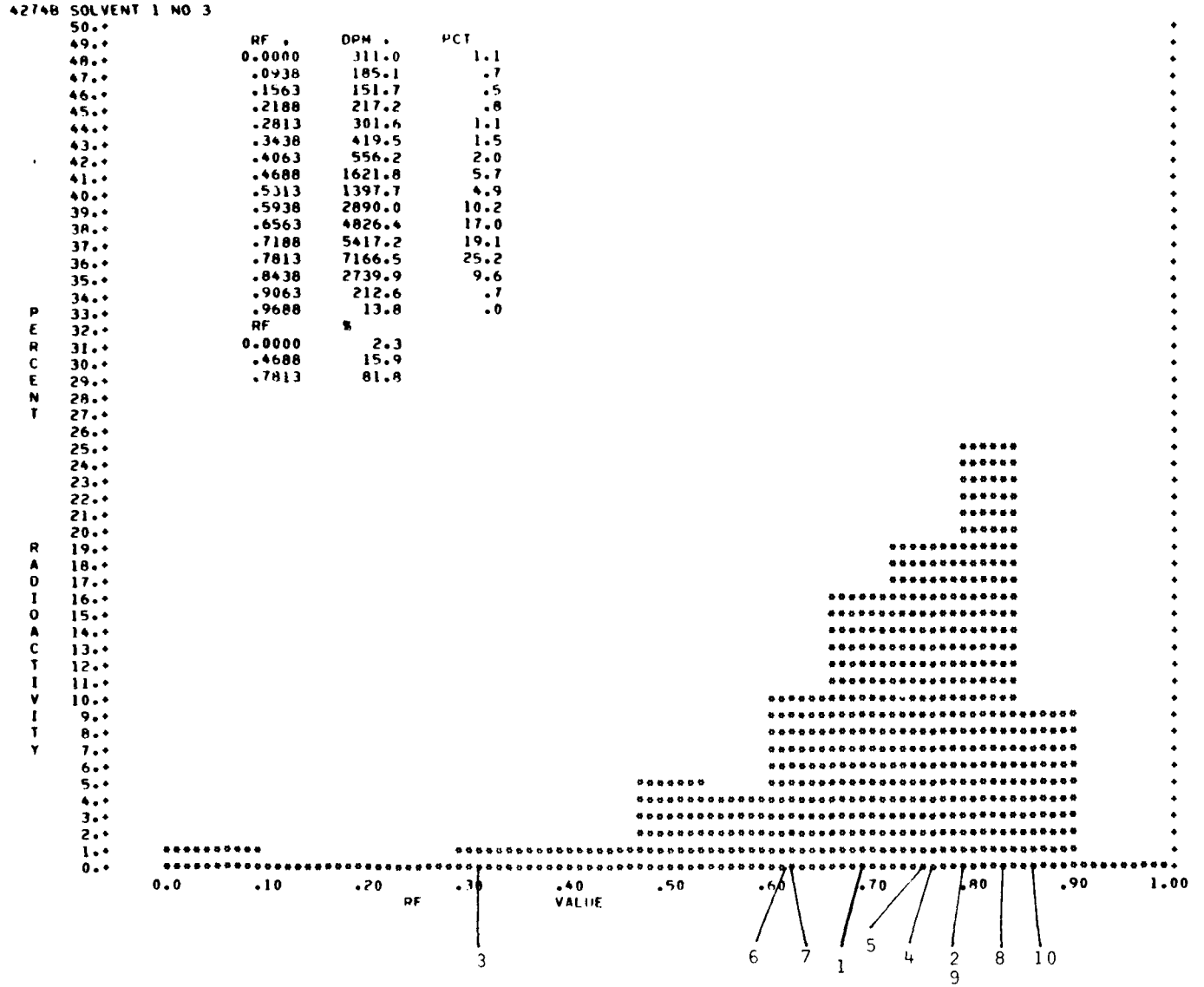

Figure 16-a-I: Oral Treatment, Incubation with Water, Solvent I

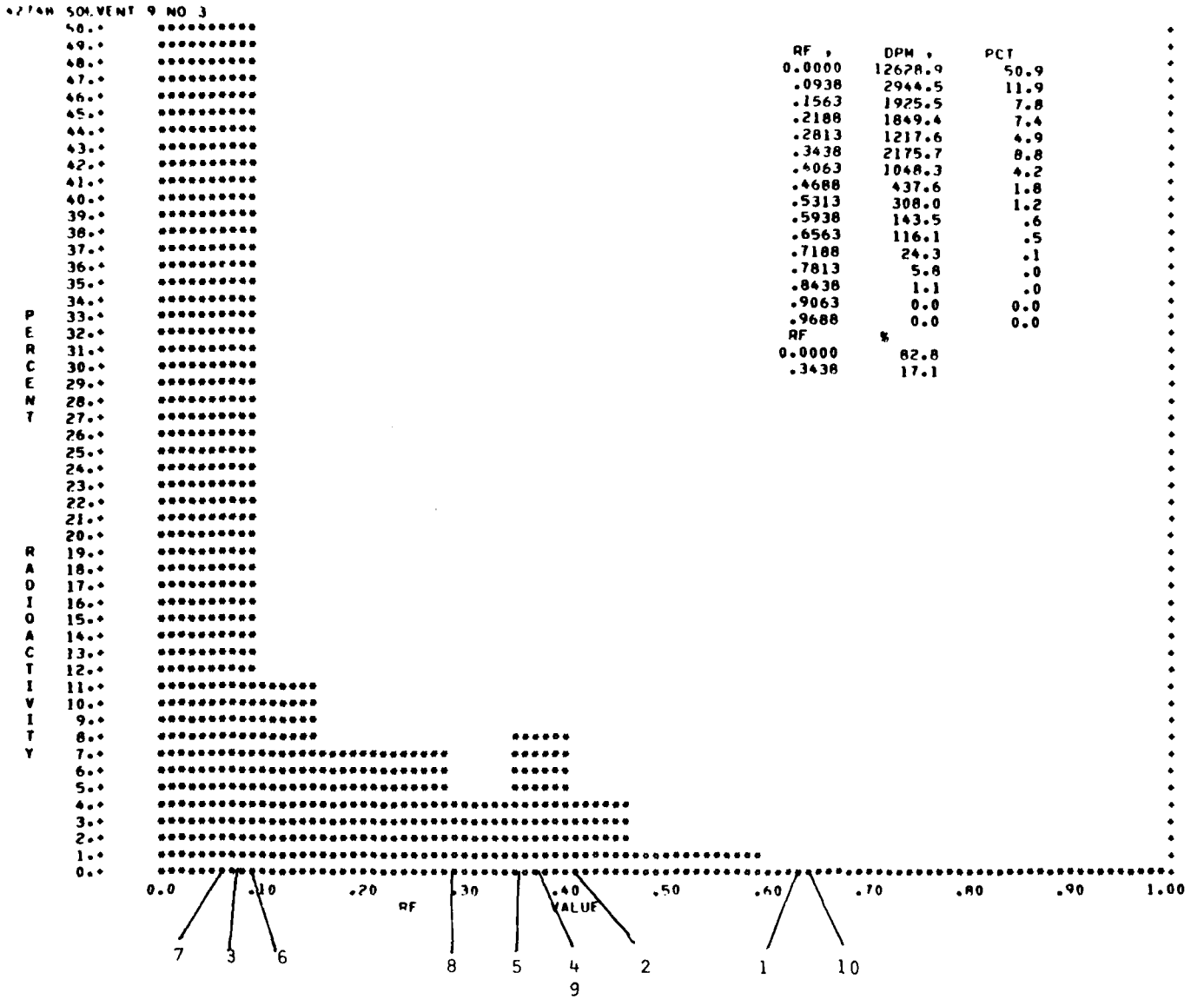

Figure 16-a-IX: Oral Treatment, Incubation with Water, Solvent IX

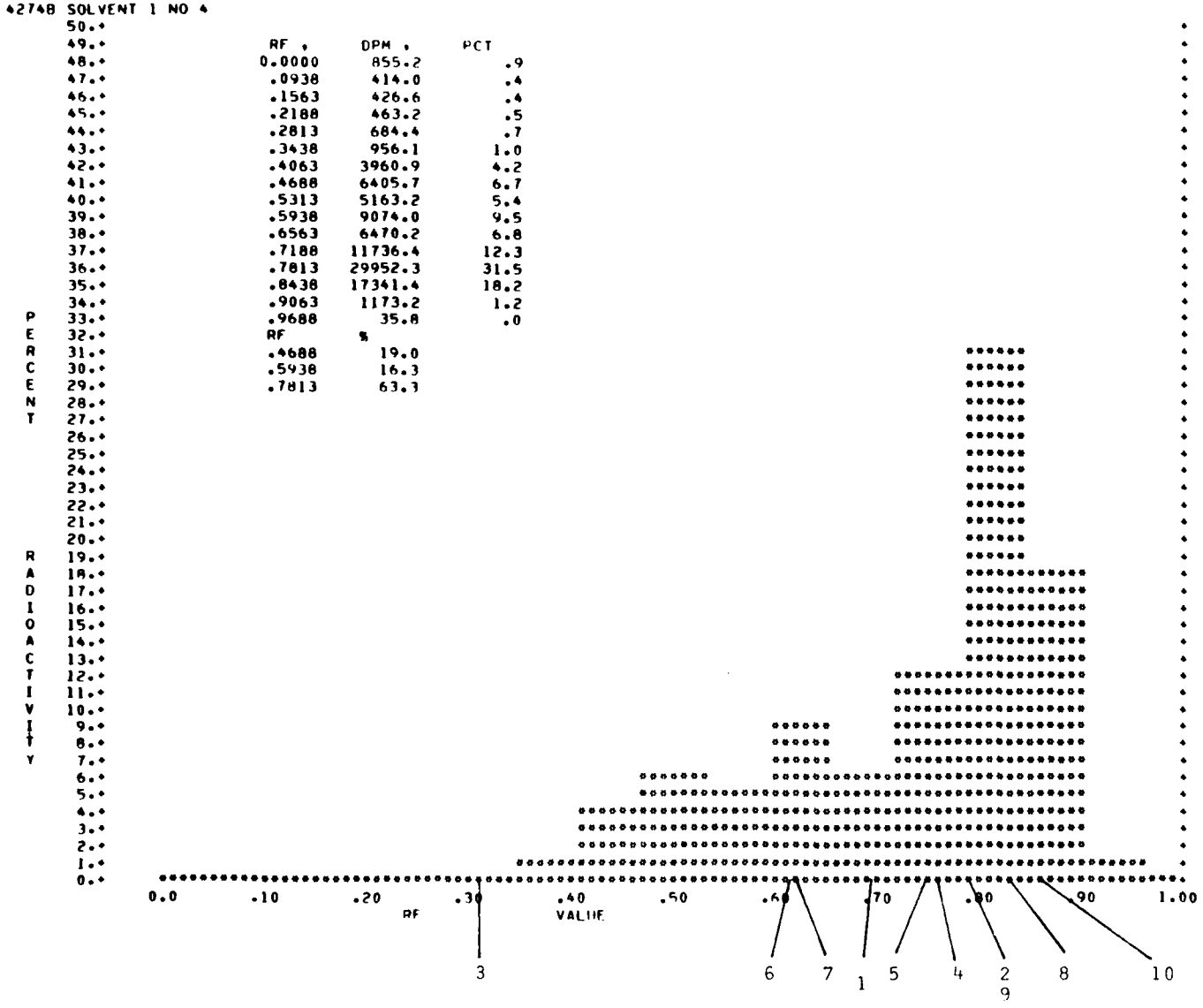

Figure 16-b-I: Oral Treatment, Incubation with β-Glucuronidase, Solvent I

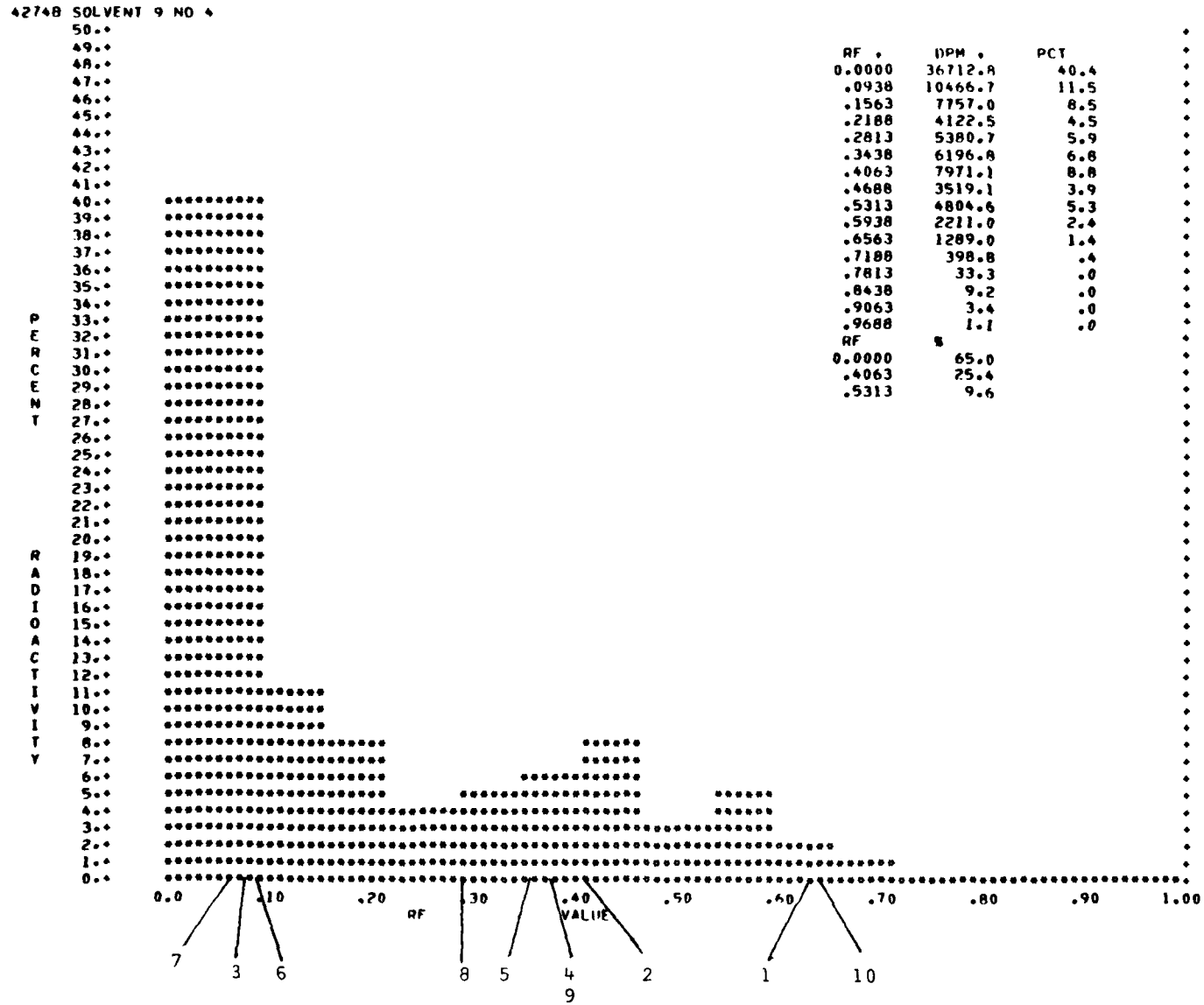

Figure 16-b-IX: Oral Treatment, Incubation with β-Glucuronidase, Solvent IX

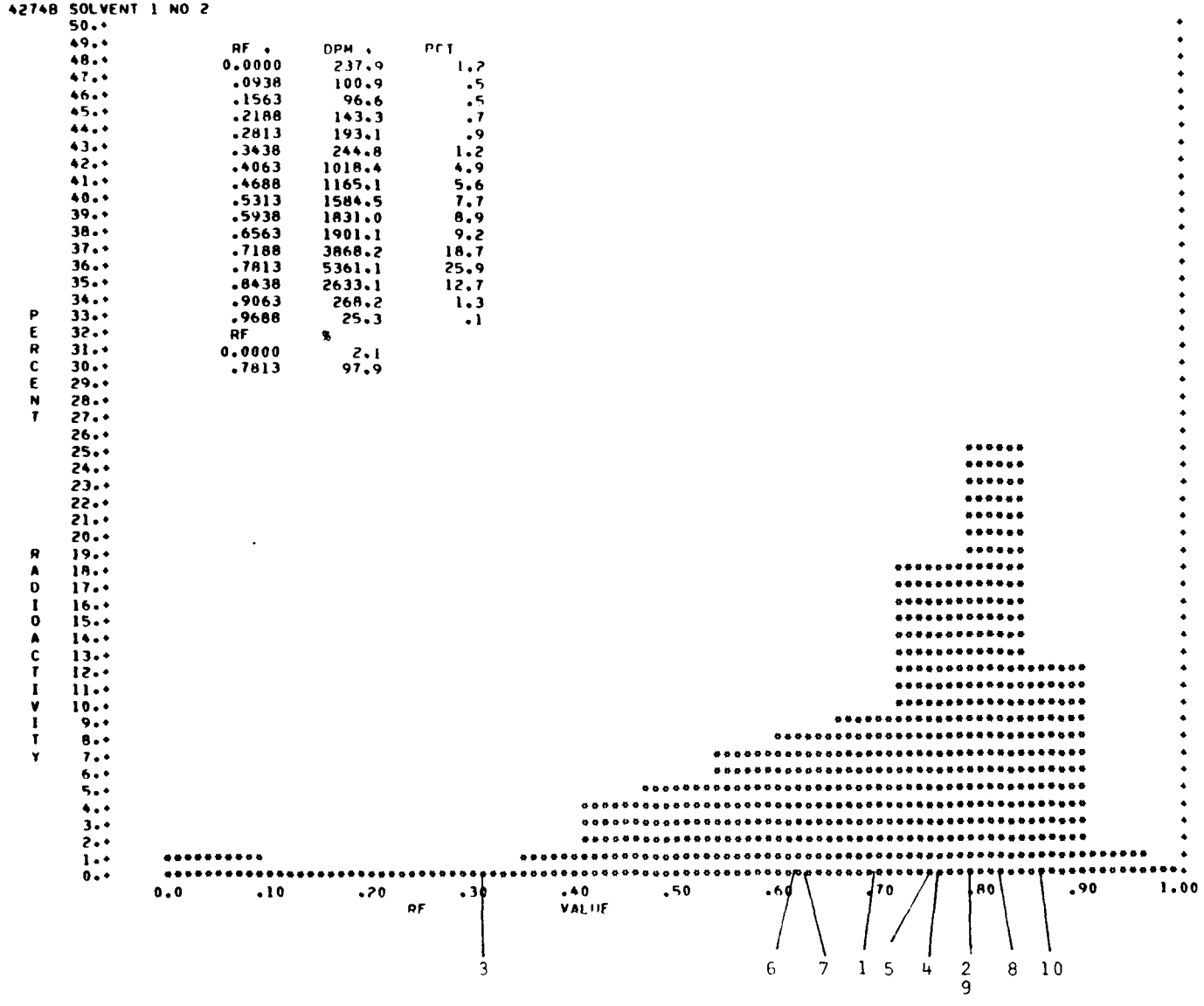

Figure 16-c-I: Dermal Application, Incubation with Water, Solvent I

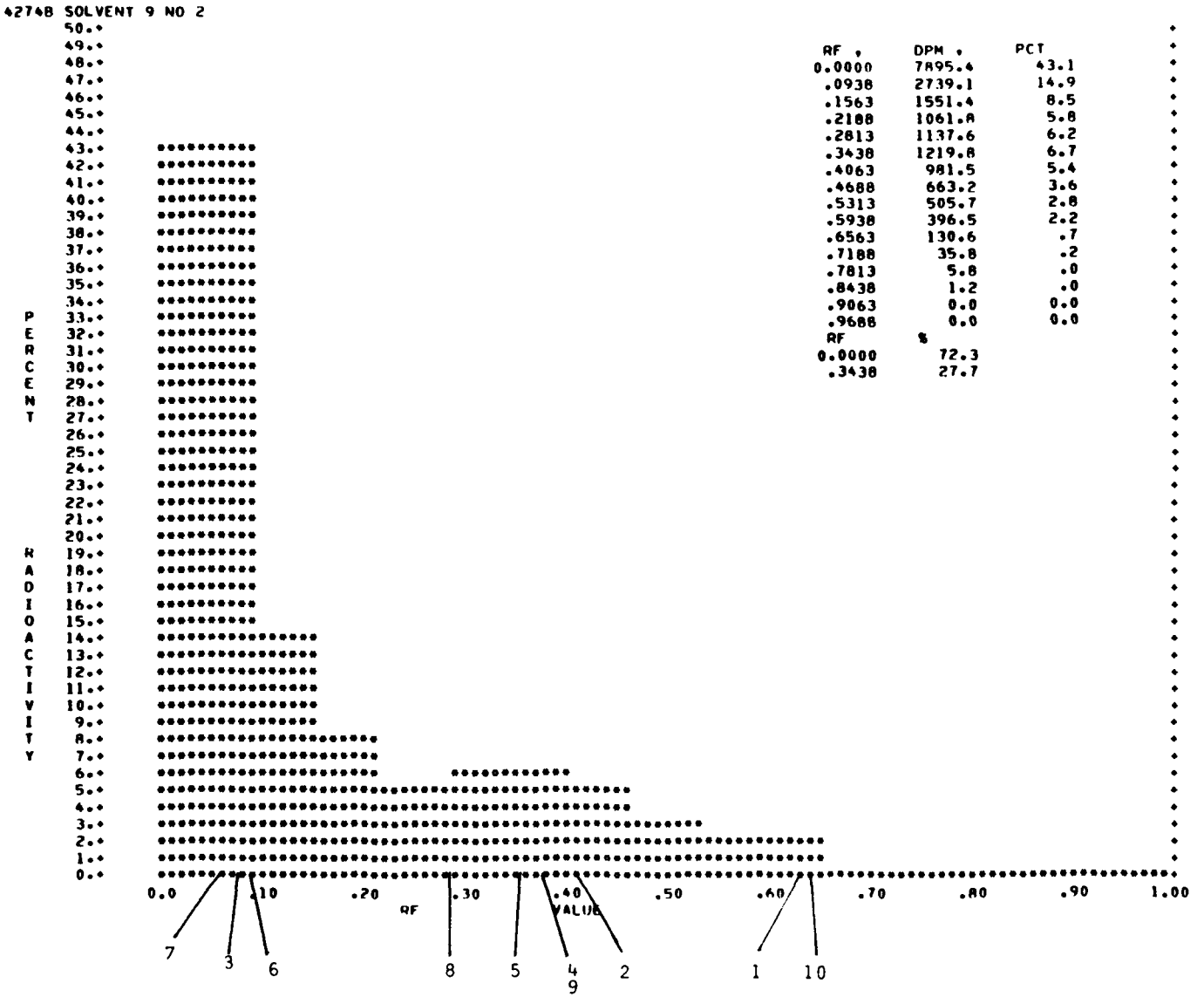

Figure 16-c-IX: Dermal Application, Incubation with Water, Solvent IX

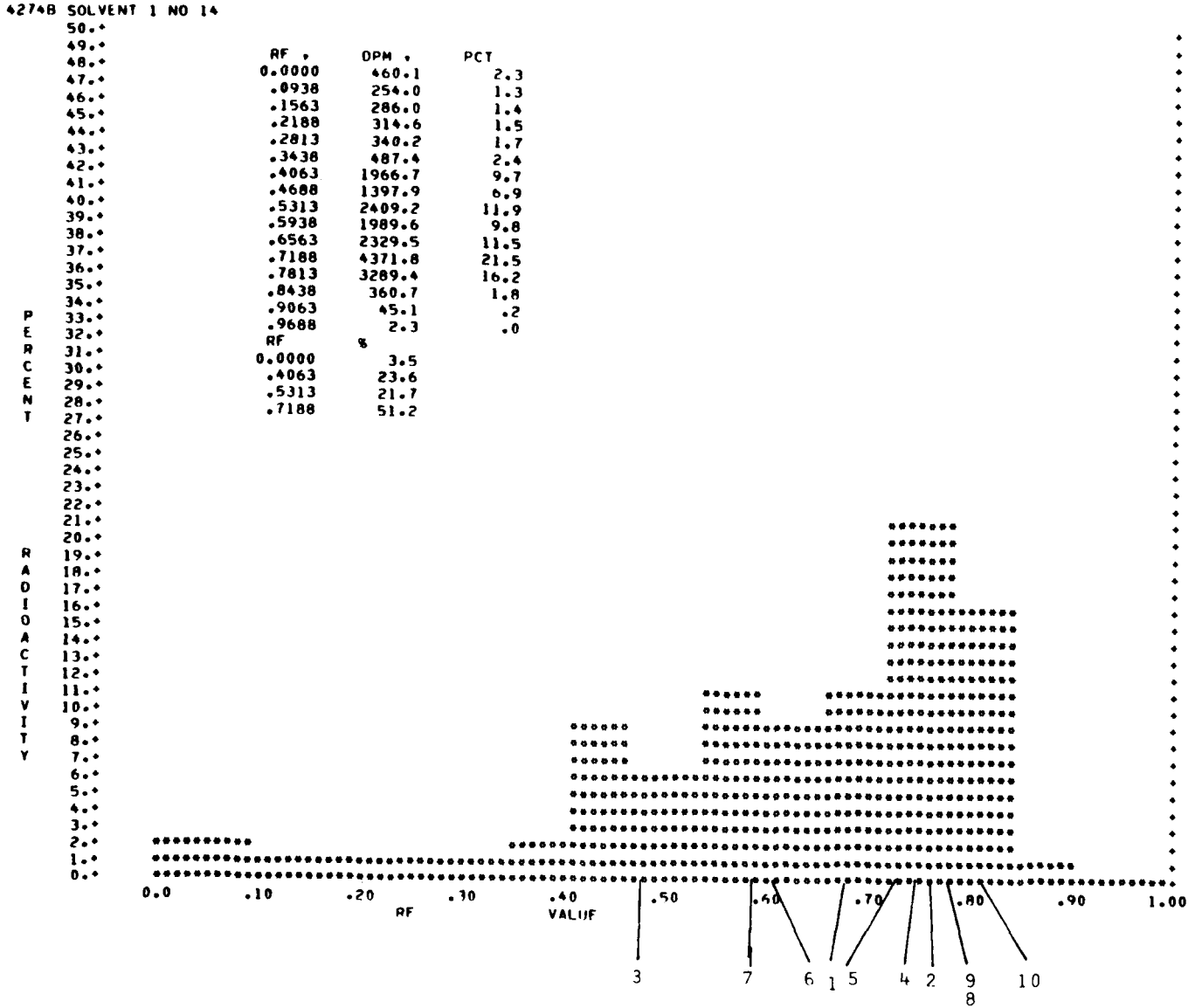

Figure 16-d-I: Dermal Application, Incubation with β-Glucuronidase, Solvent I

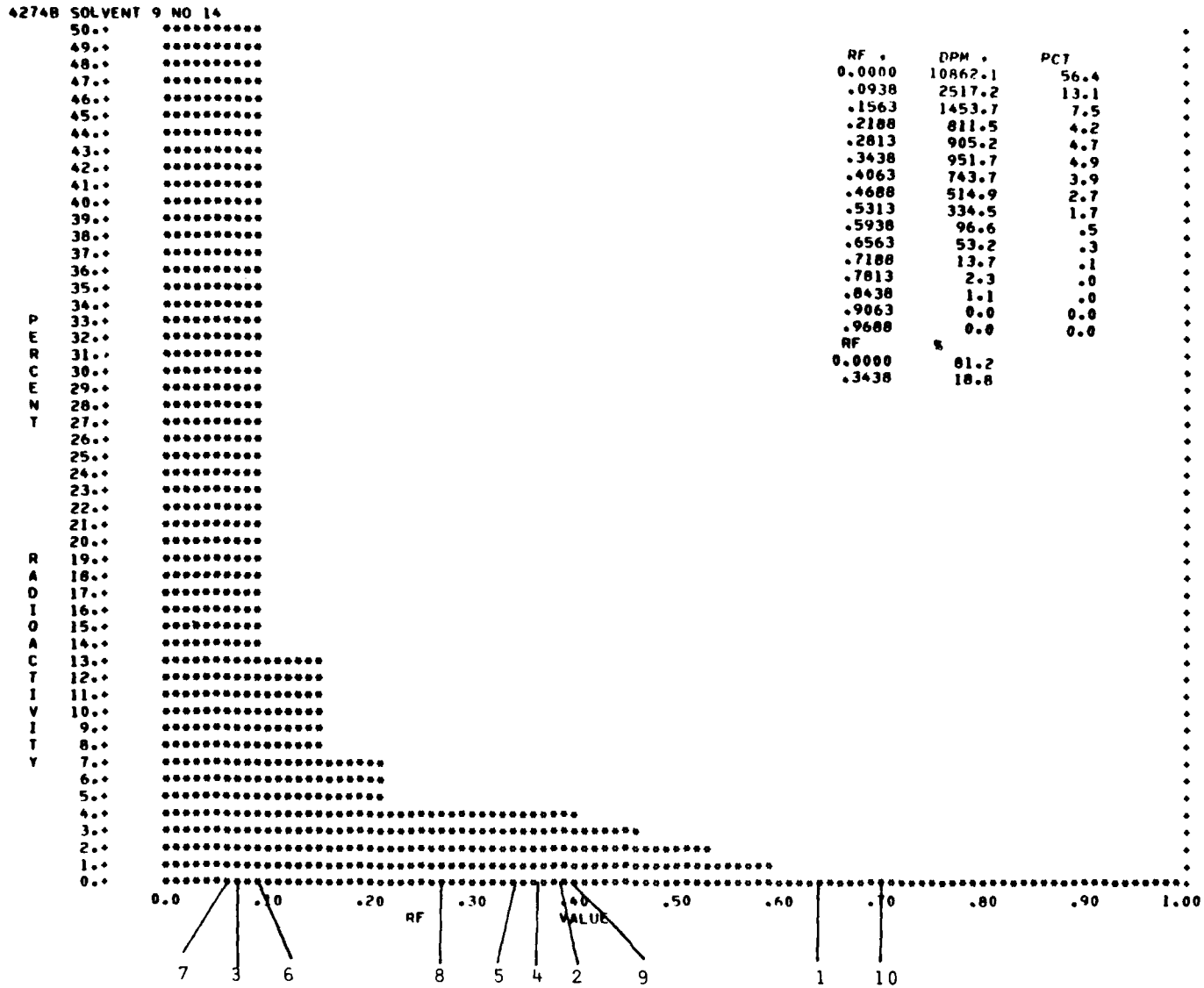

Figure 16-d-IX: Dermal Application, Incubation with β-Glucuronidase, Solvent IX

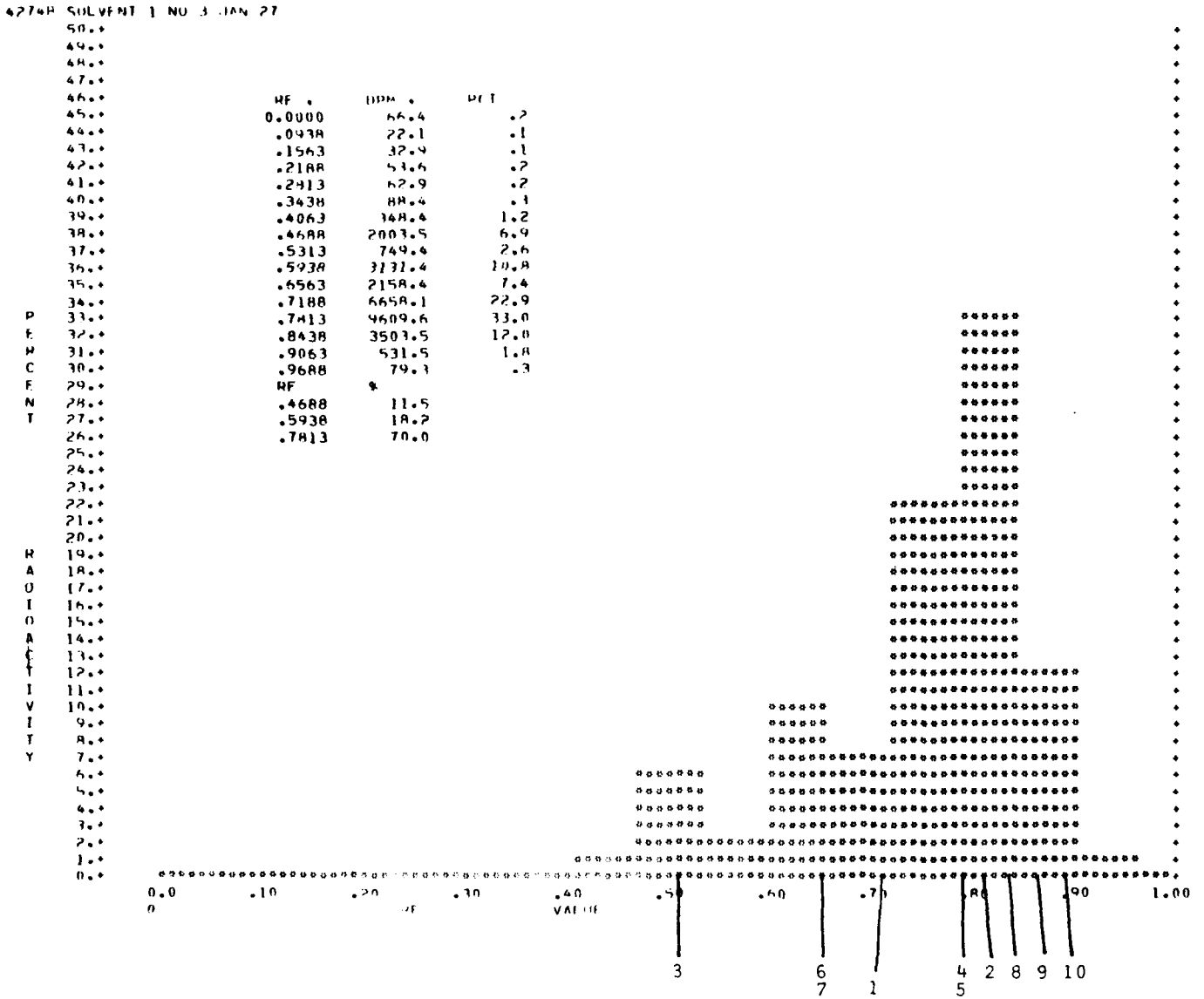

Figure 16-e-I: Oral Treatment, Incubation with Water, Solvent I

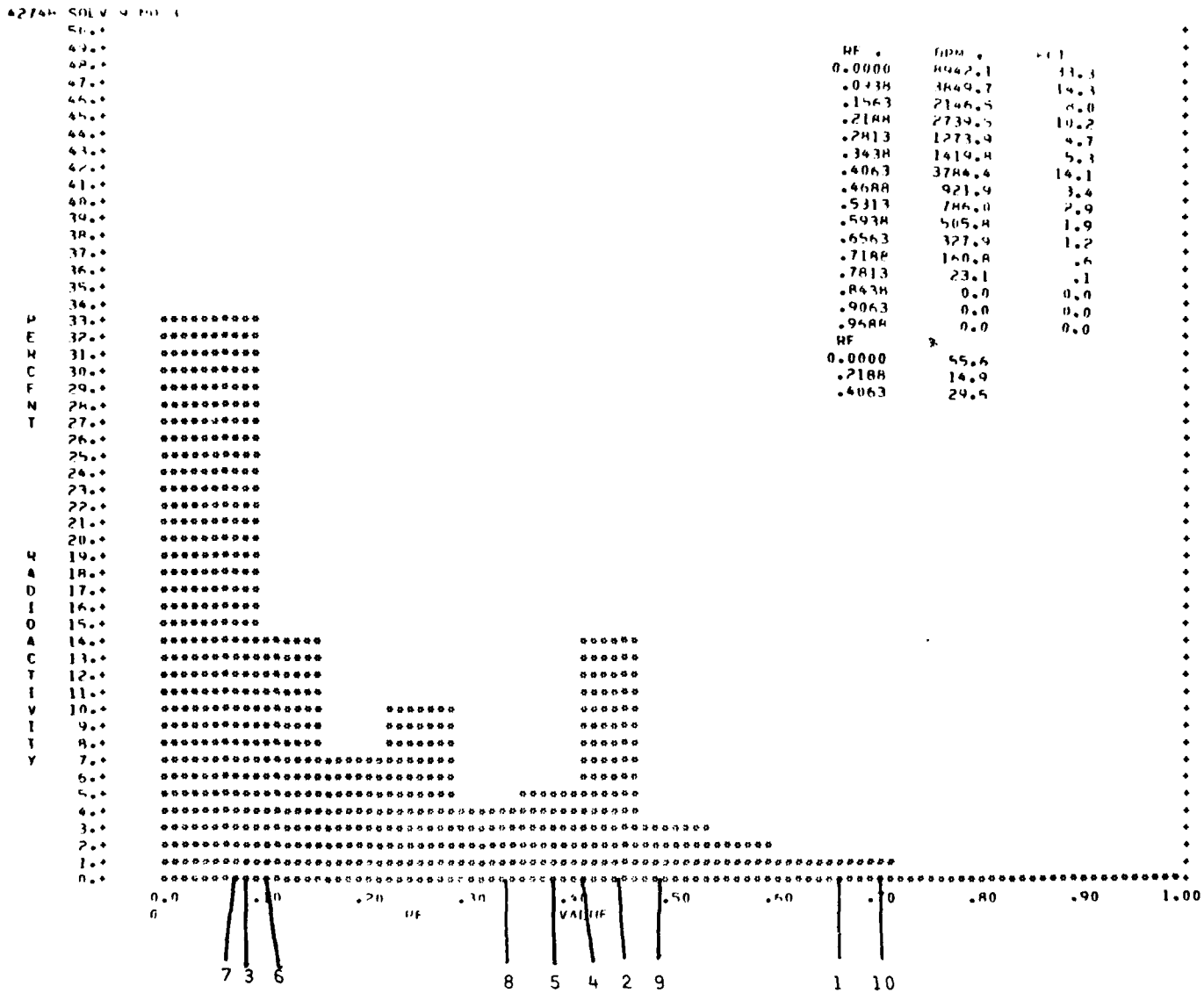

Figure 16-e-IX: Oral Treatment, Incubation with Water, Solvent IX

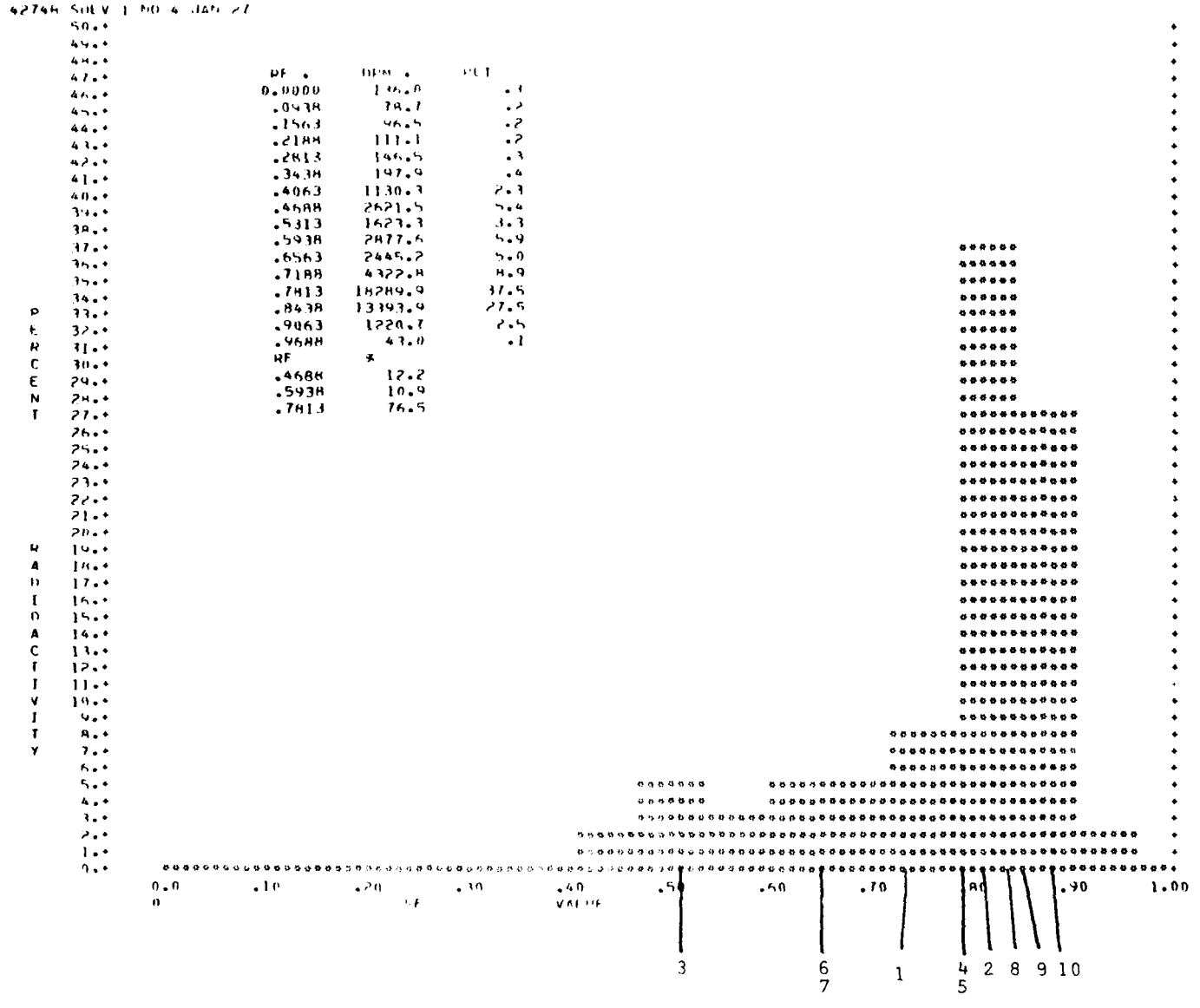

Figure 16-f-I: Oral Treatment, Incubation with β-Glucuronidase, Solvent I

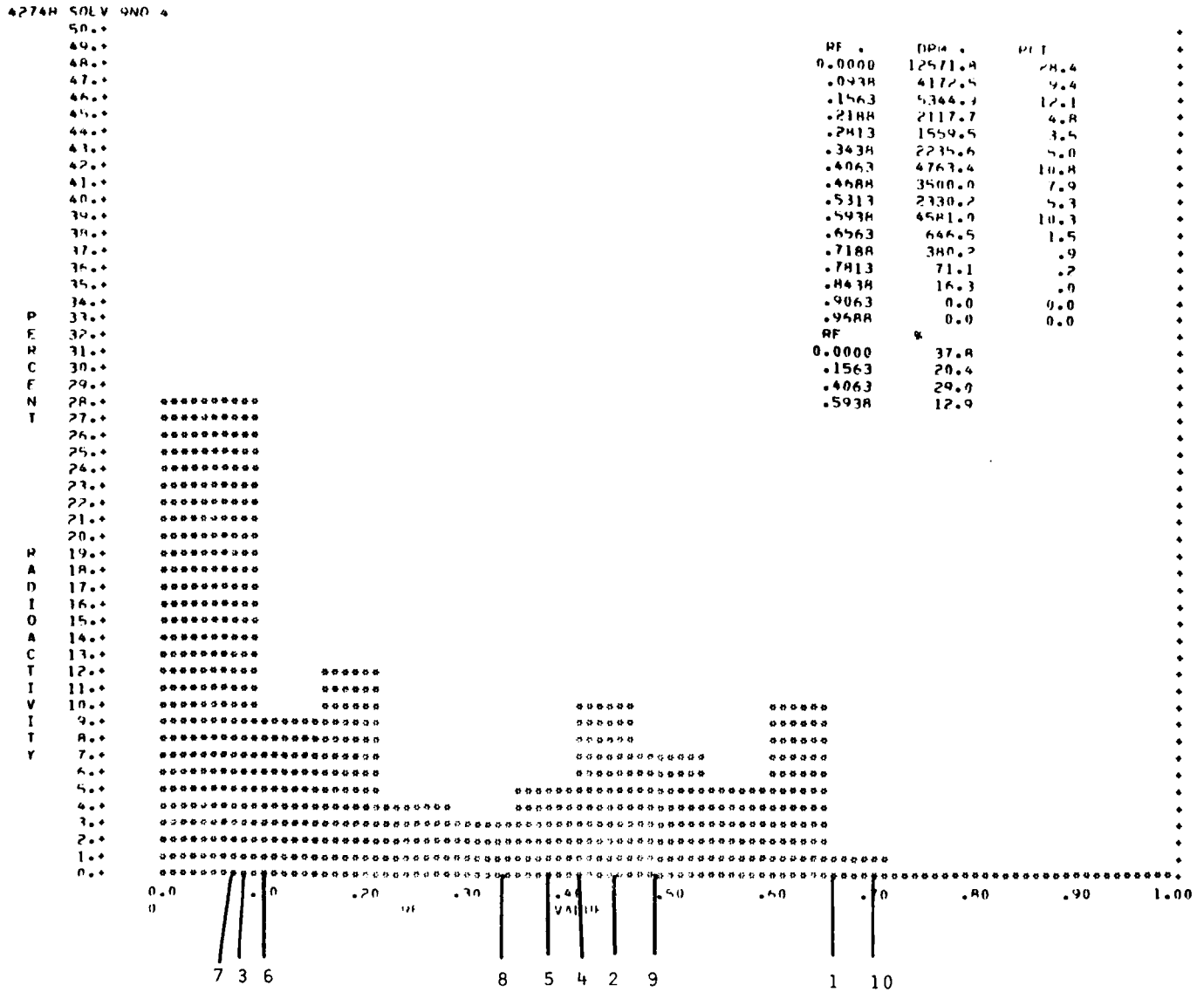

Figure 16-f-IX: Oral Treatment, Incubation with β-Glucuronidase, Solvent IX

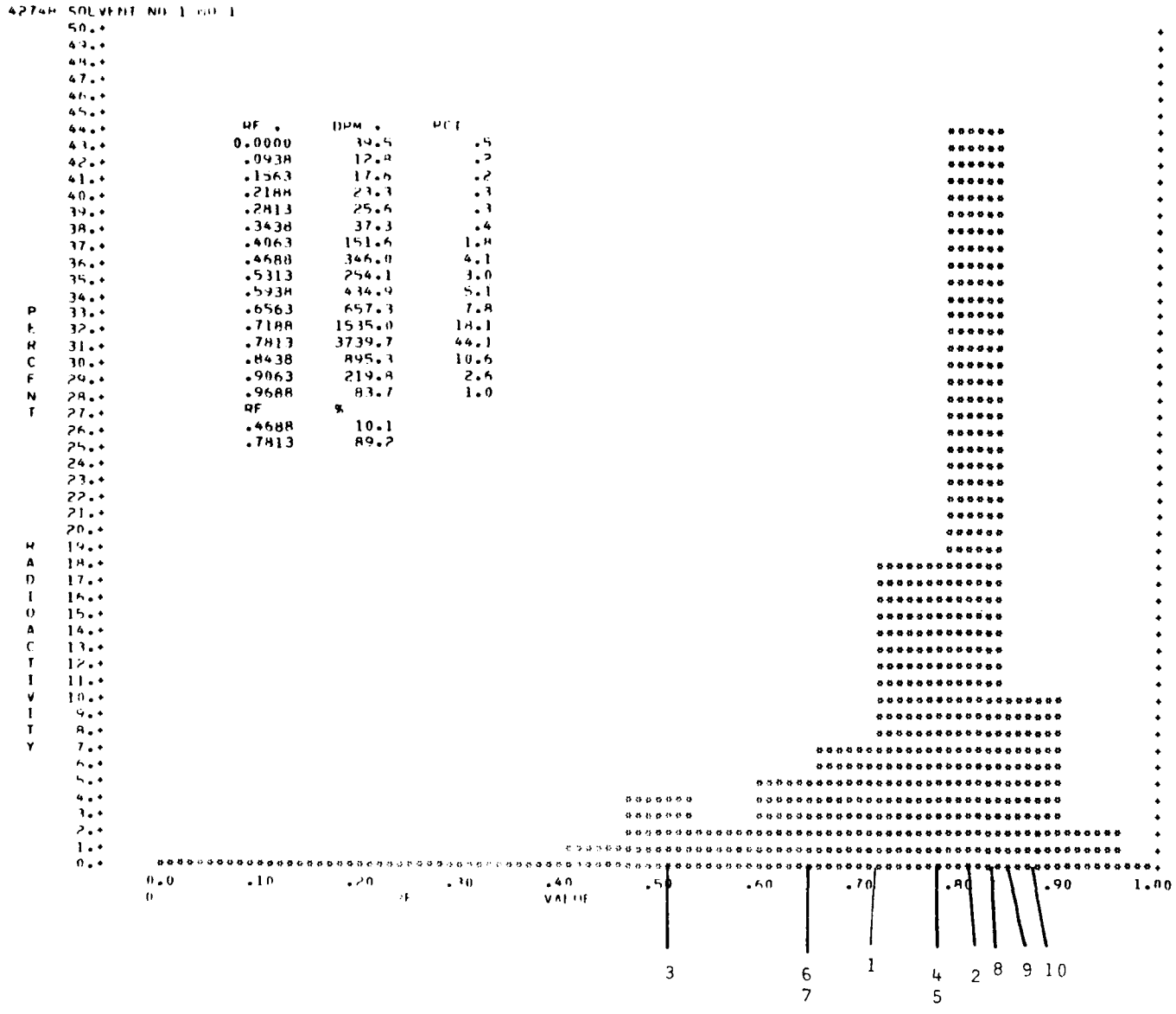

Figure 16-g-I: Dermal Application, Incubation with Water, ß-Glucuronidase, Solvent I

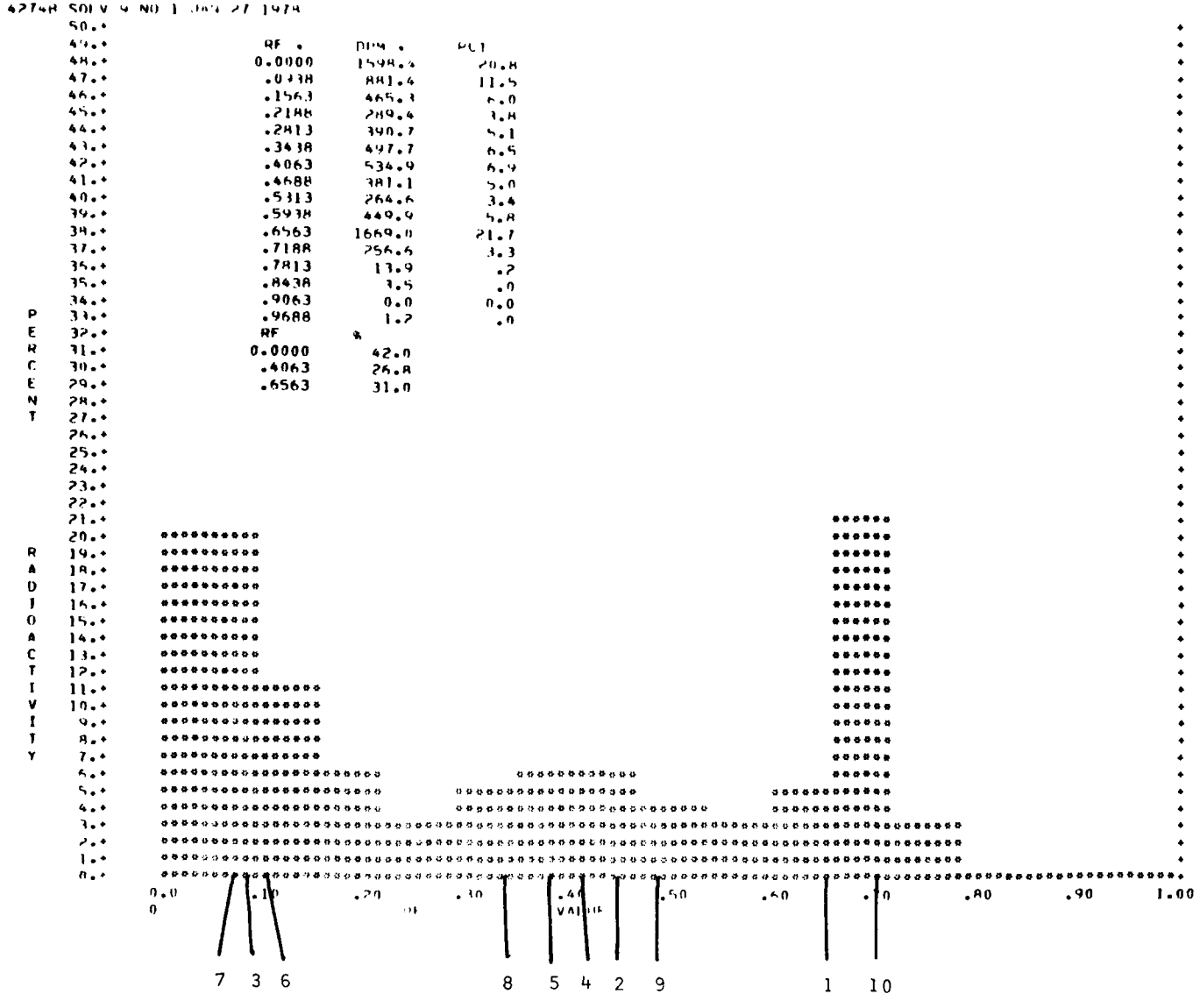

Figure 16-g-IX: Dermal Application, Incubation with Water, β-Glucuronidase, Solvent I

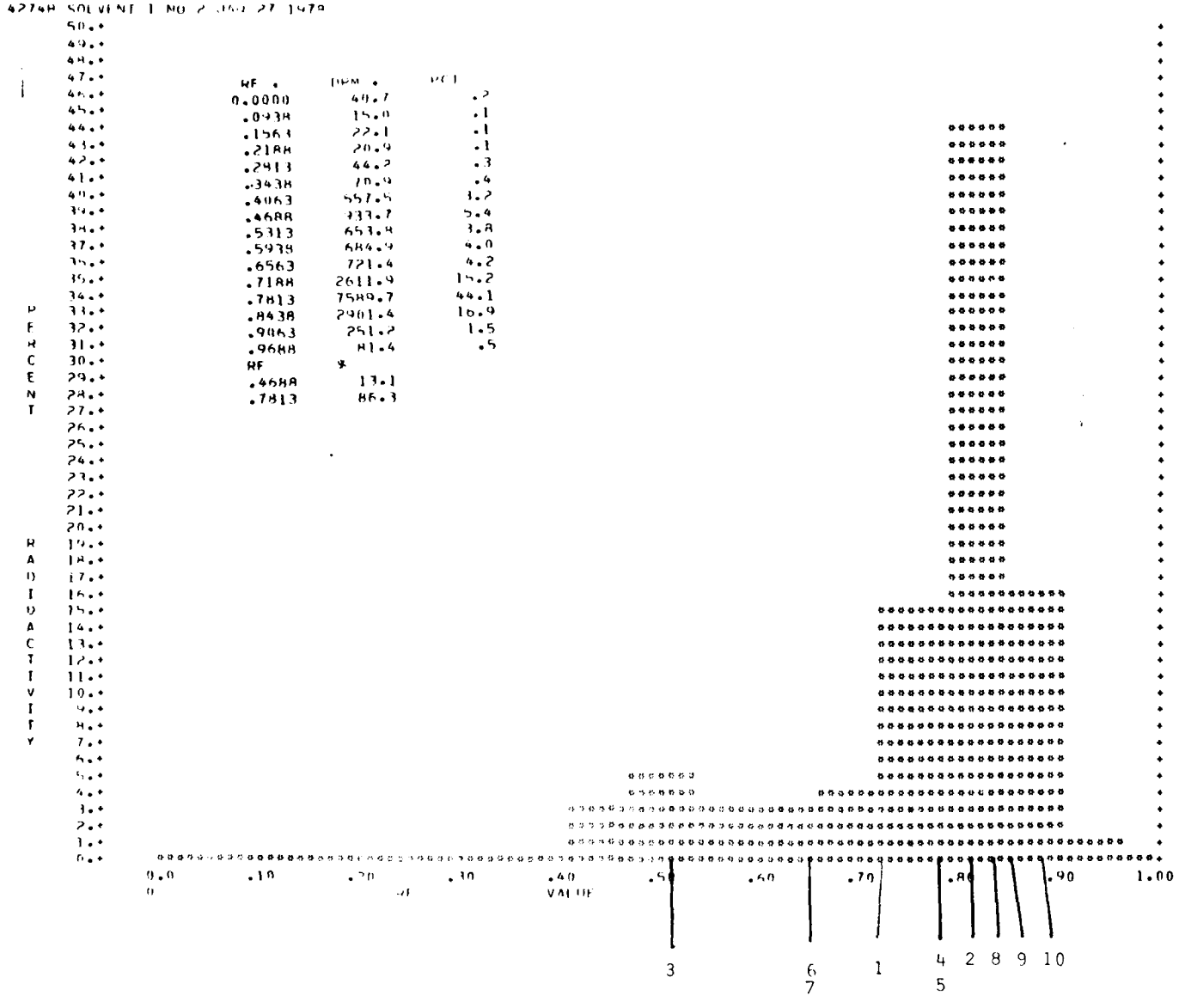

Figure 16-h-I: Dermal Application, Incubation with β-Glucuronidase, Solvent I

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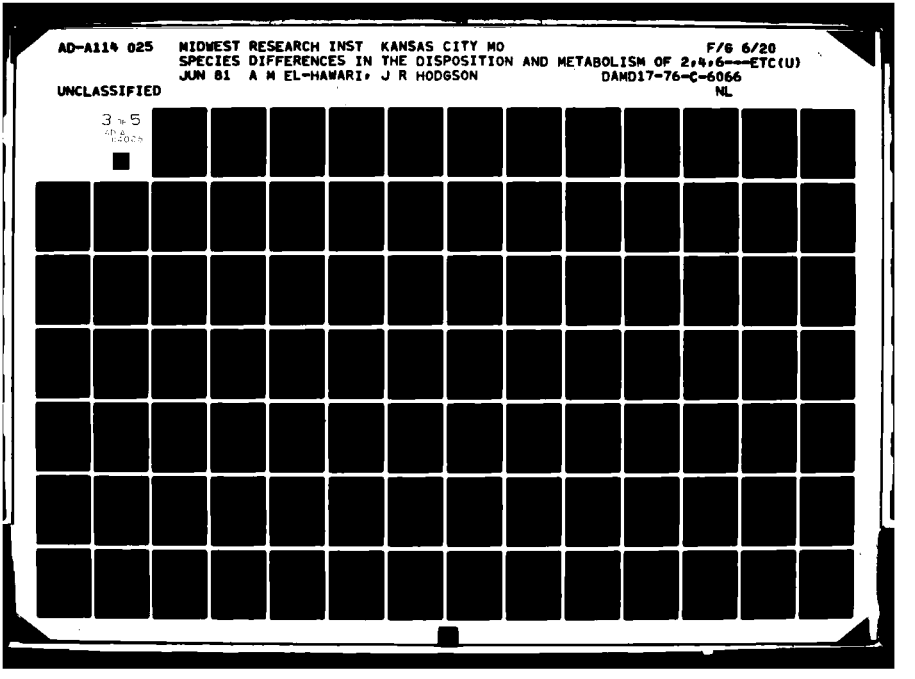

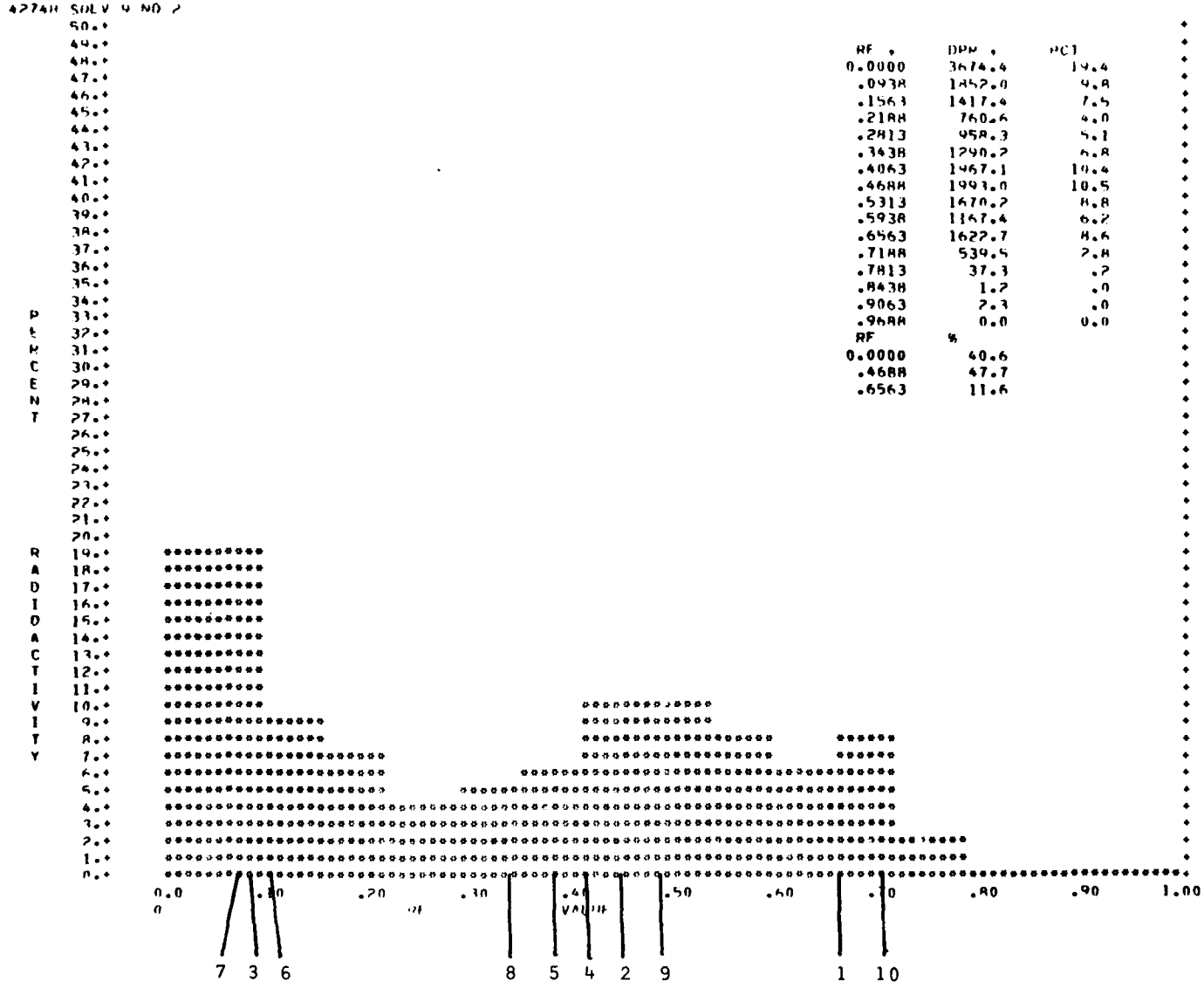

Figure 16-h-IX: Dermal Application, Incubation with β-Glucuronidase, Solvent IX

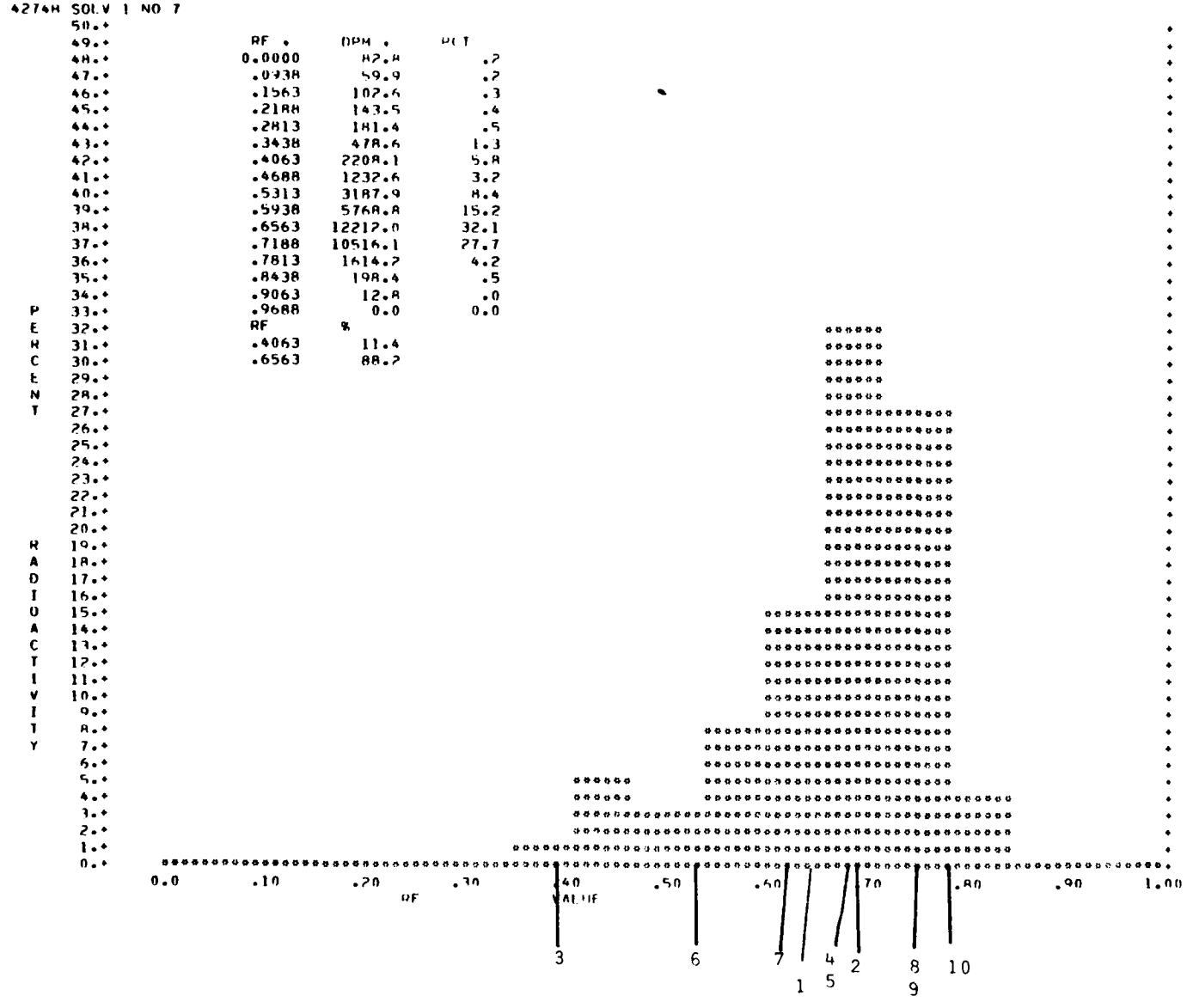

Figure 16-k-I: Oral Treatment, Incubation with Water, Solvent I

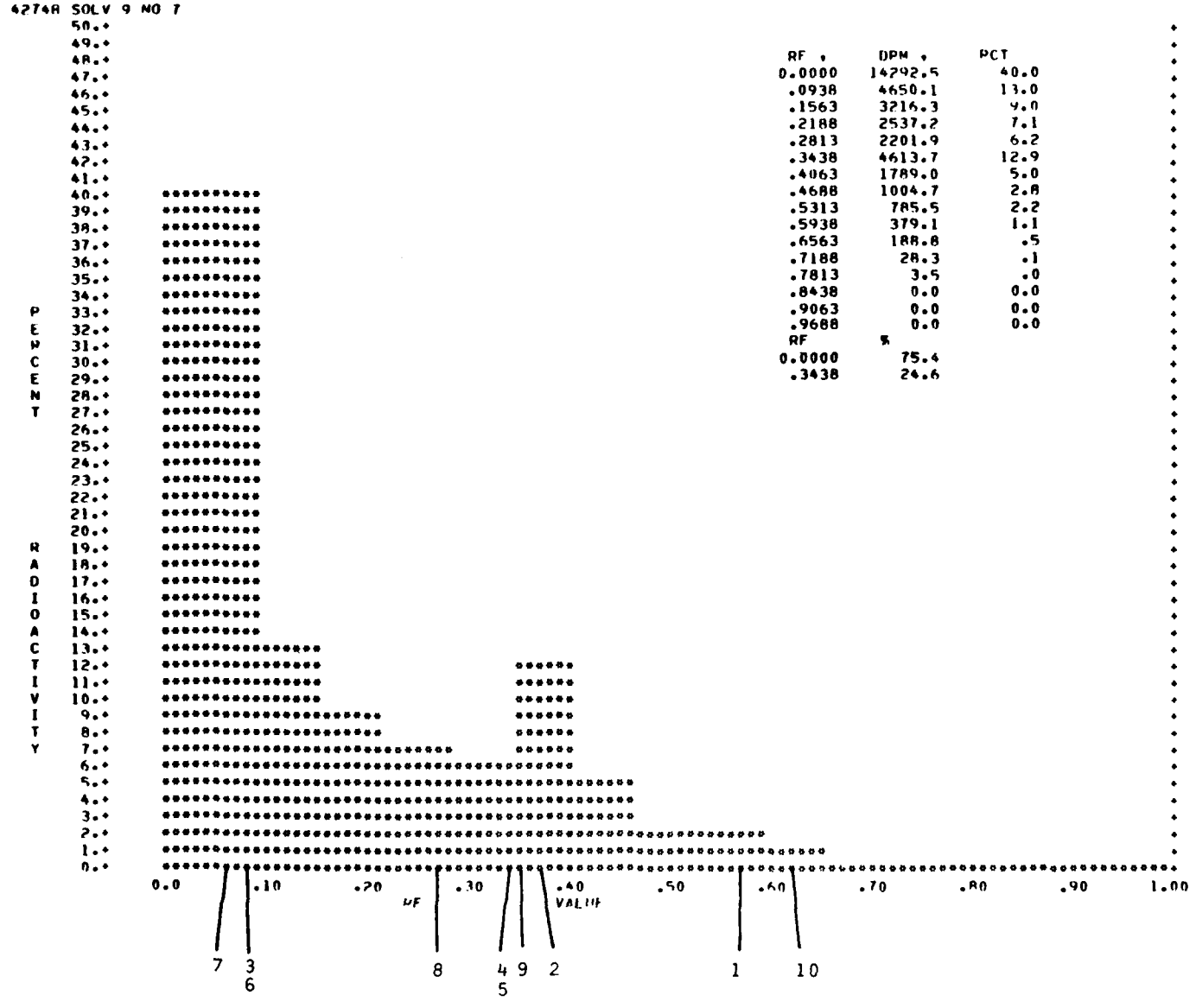

Figure 16-k-IX: Oral Treatment, Incubation with Water, Solvent IX

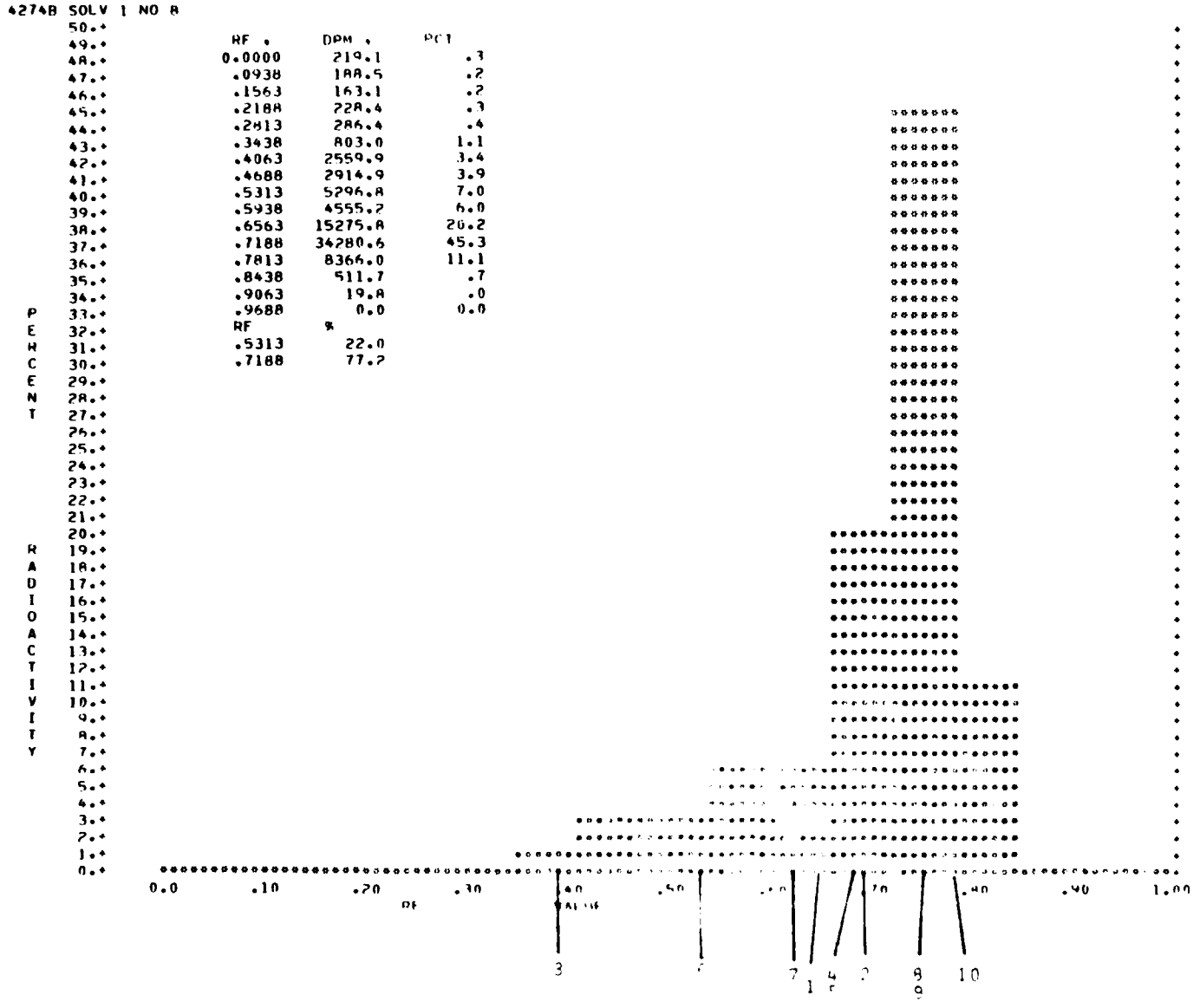

Figure 16-1-I: Oral Treatment, Incubation with β-Glucuronidase, Solvent I

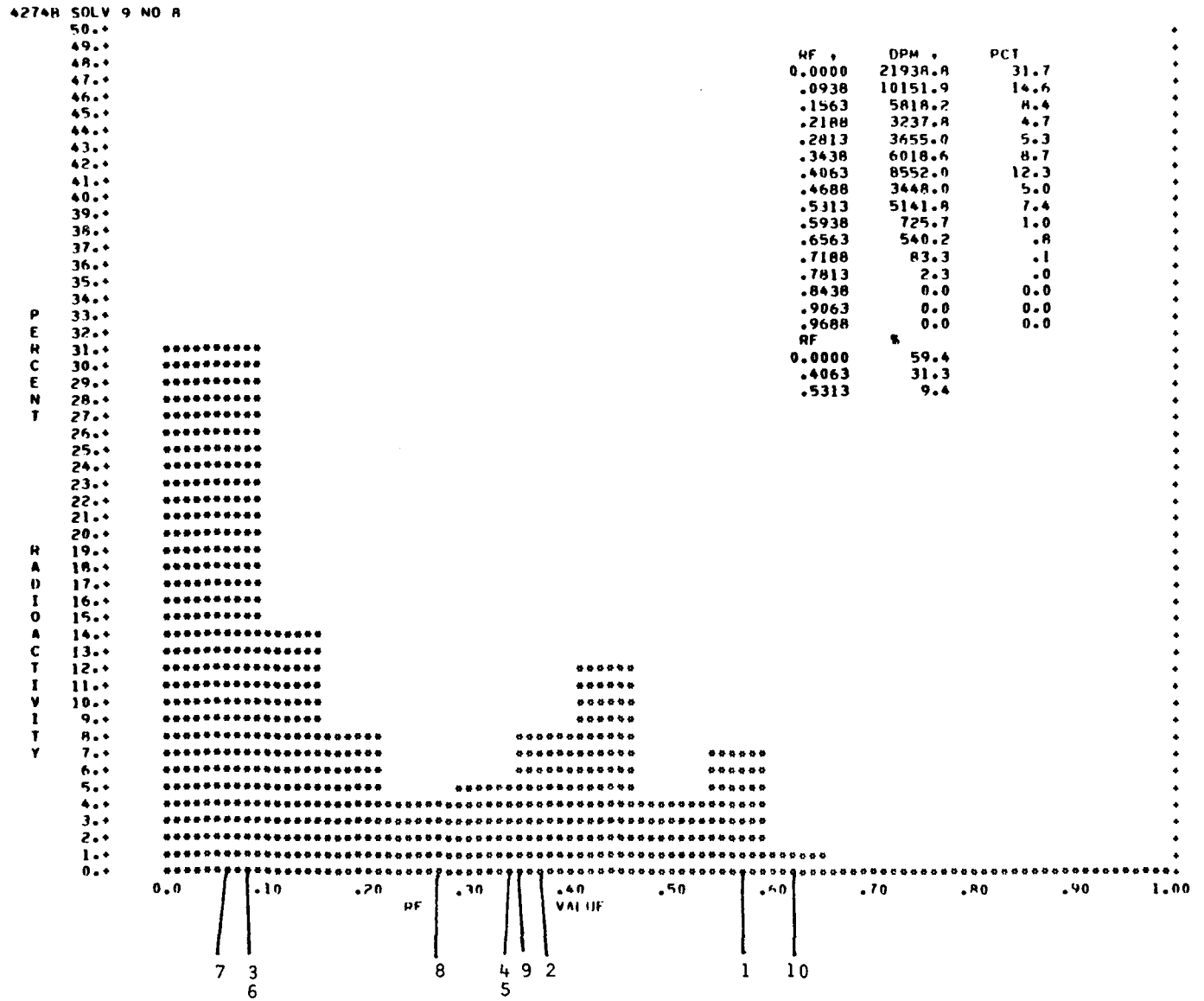

Figure 16-1-IX: Oral Treatment, Incubation with β-Glucuronidase, Solvent IX

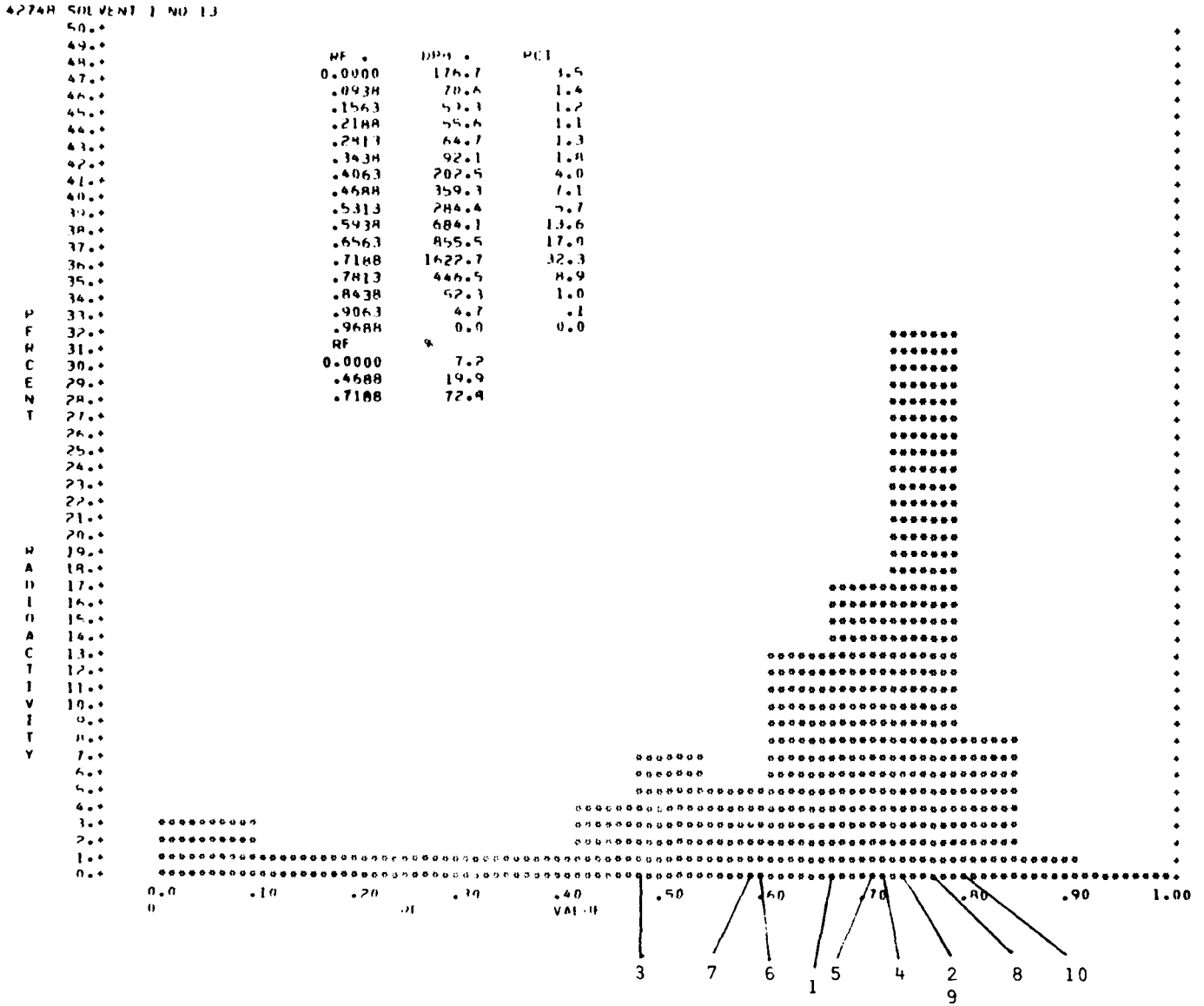

Figure 16-m-I: Dermal Application, Incubation with Water, Solvent I

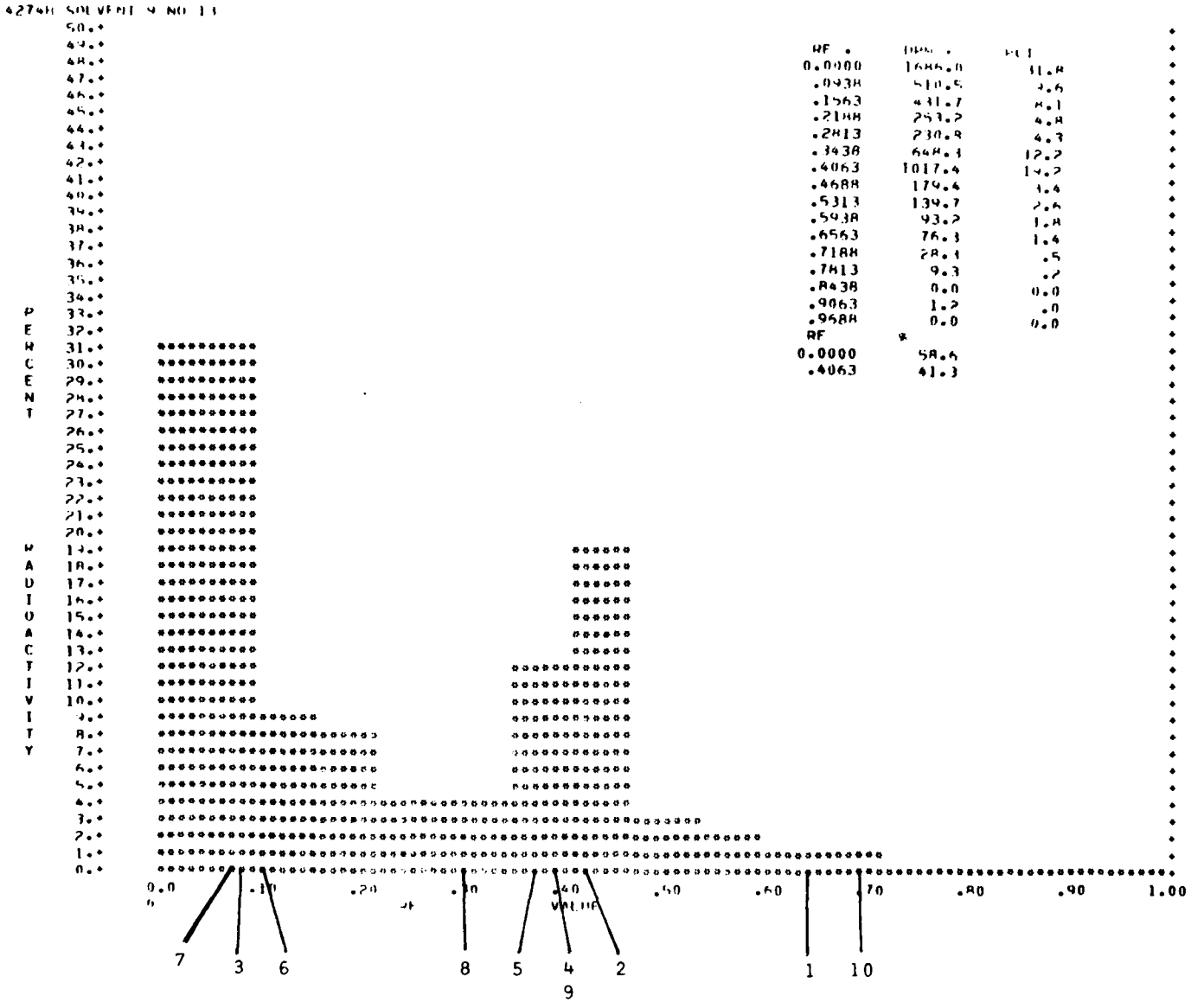

Figure 16-m-IX: Dermal Application, Incubation with Water, Solvent IX

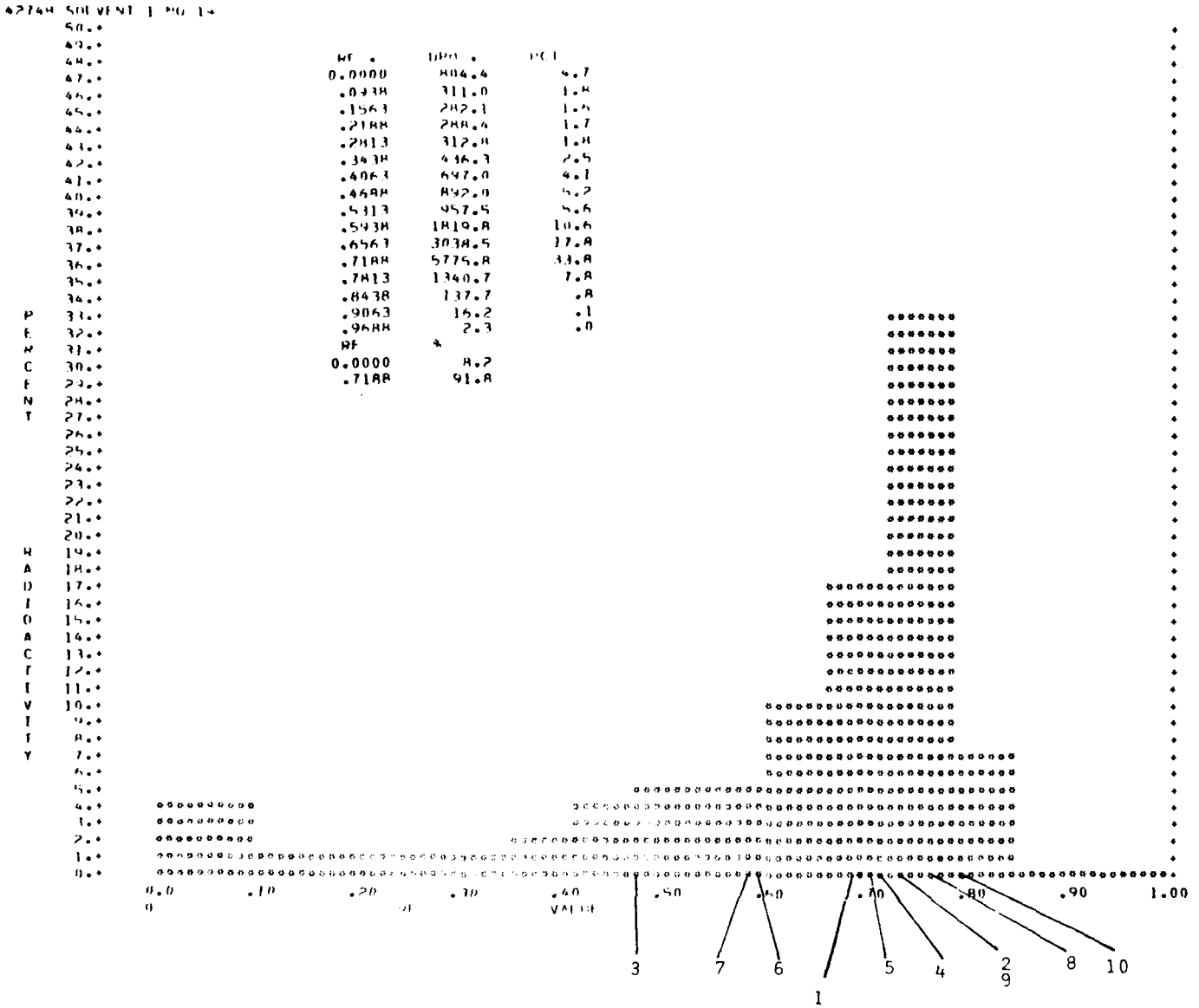

Figure 16-n-I: Dermal Application, Incubation with β-Glucuronidase, Solvent I

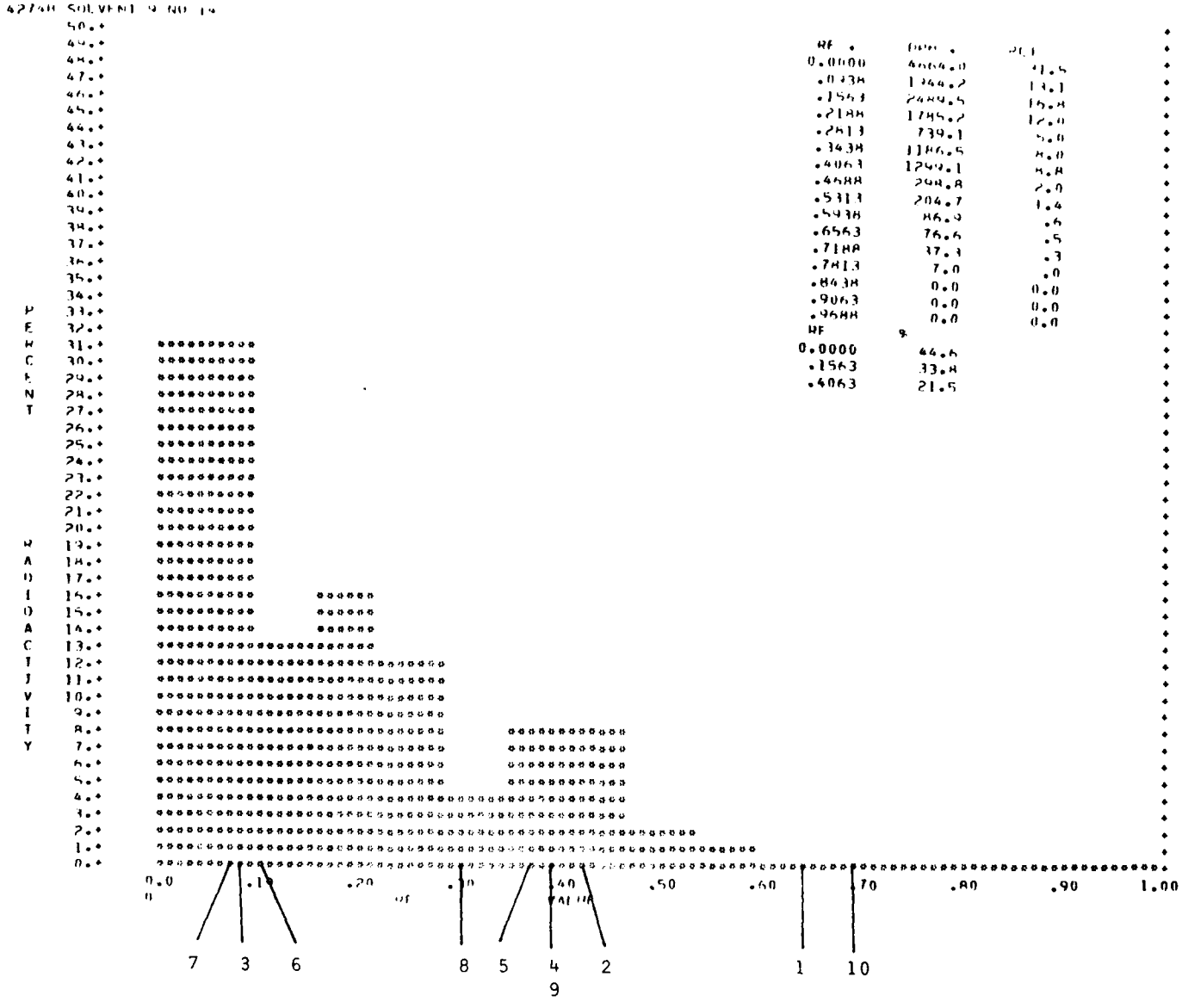

Figure 16-n-IX: Dermal Application, Incubation with β-Glucuronidase, Solvent IX

Figure 17: TLC of Ethyl Acetate-Extractable Products Obtained from 24-Hr Urine of Female Rats Treated Orally or Dermally with ^{14}C-TNT. Samples of urine were incubated with acetate buffer and β-glucuronidase before extraction with ethyl acetate. Incubation with acetate buffer and water served as control. Reference standards are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzylalcohol
3. Trinitrobenzoic Acid
4. 4-Amino-2,6-Dinitrotoluene
5. 2-Amino-4,6-Dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2,6'-Tetranitro-4,4'-azoxytoluene

Figure 17 follows

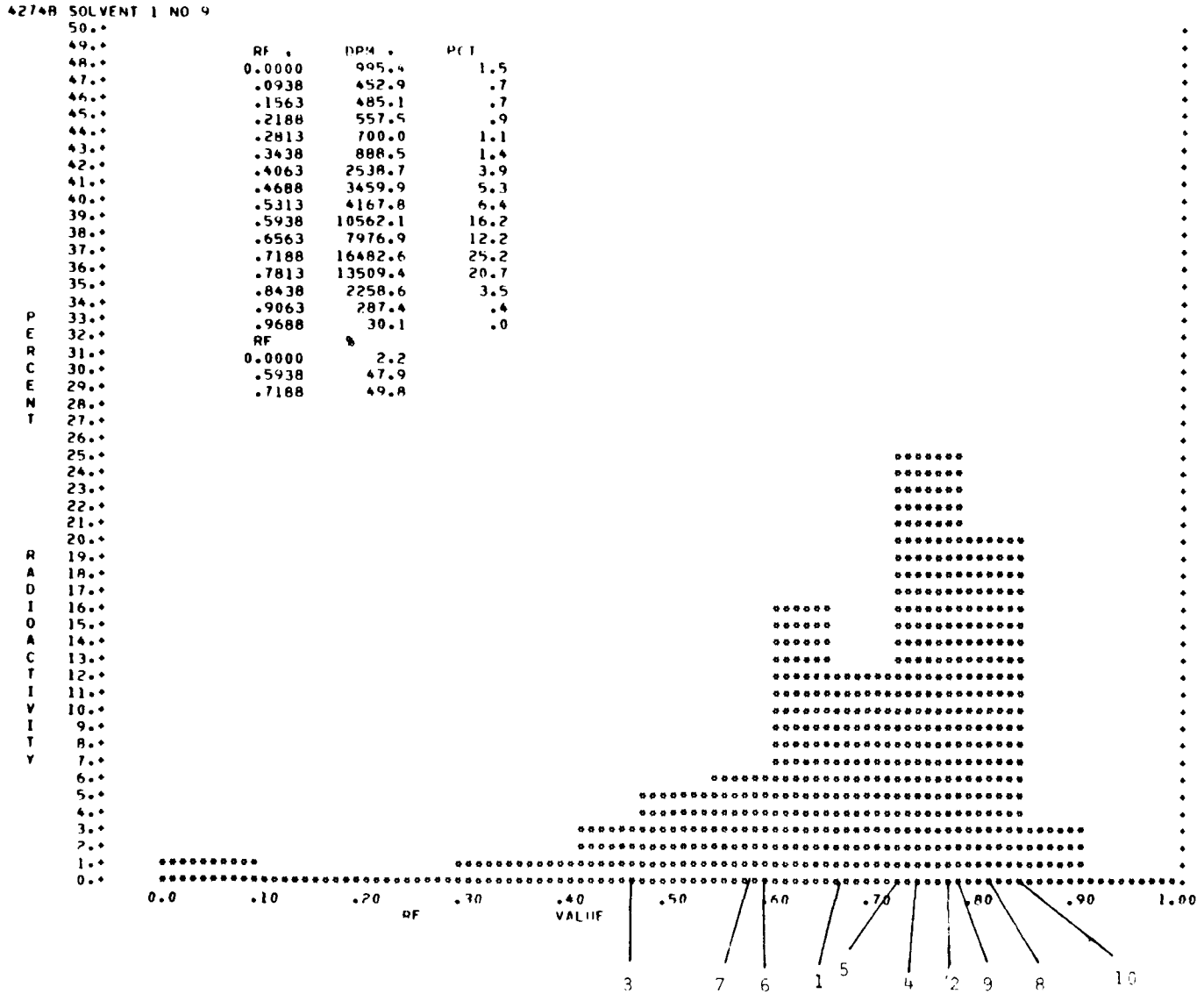

Figure 17-a-I: Oral Treatment, Incubation with Water, Solvent I.

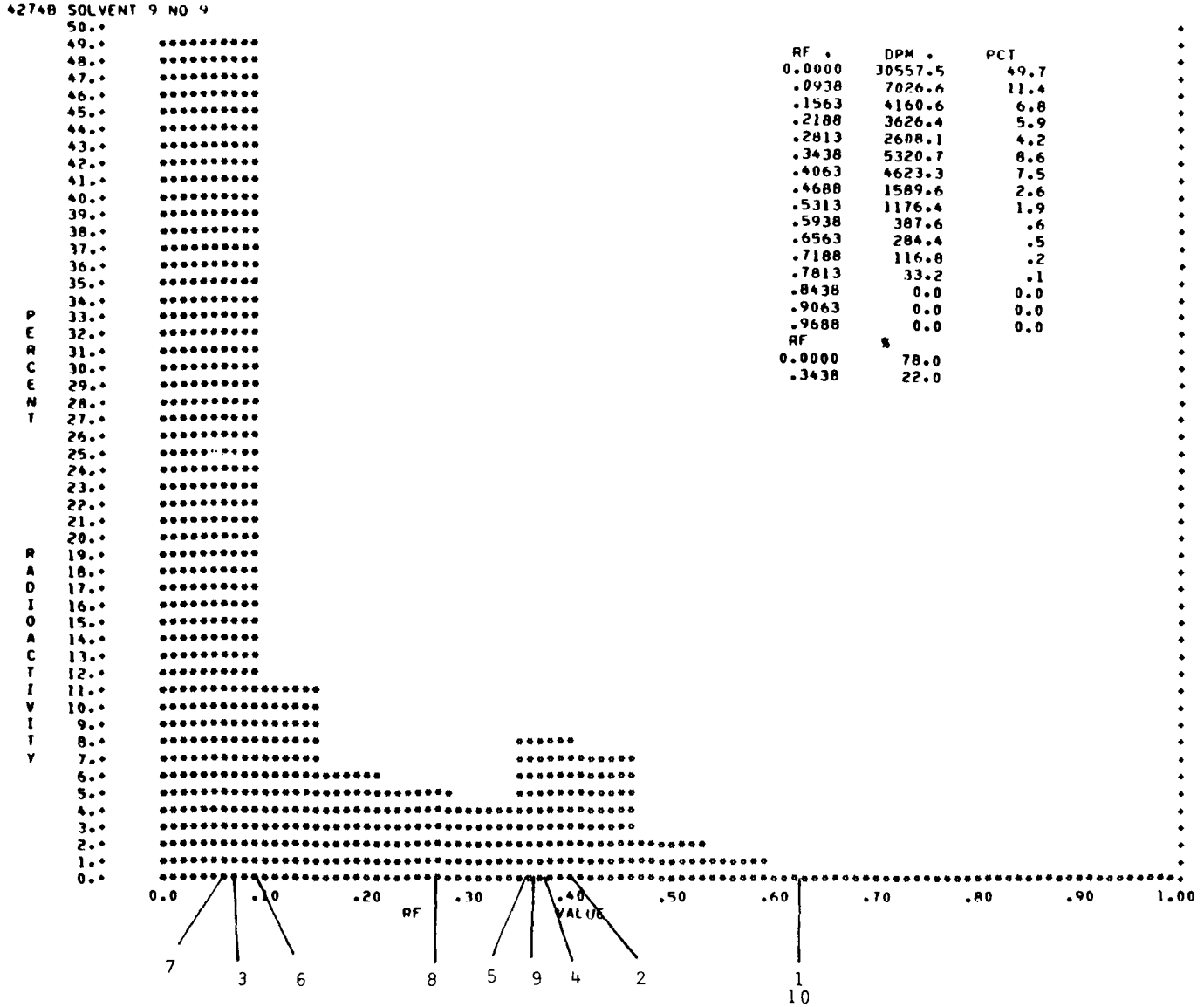

Figure 17-a-IX: Oral Treatment, Incubation with Water, Solvent IX.

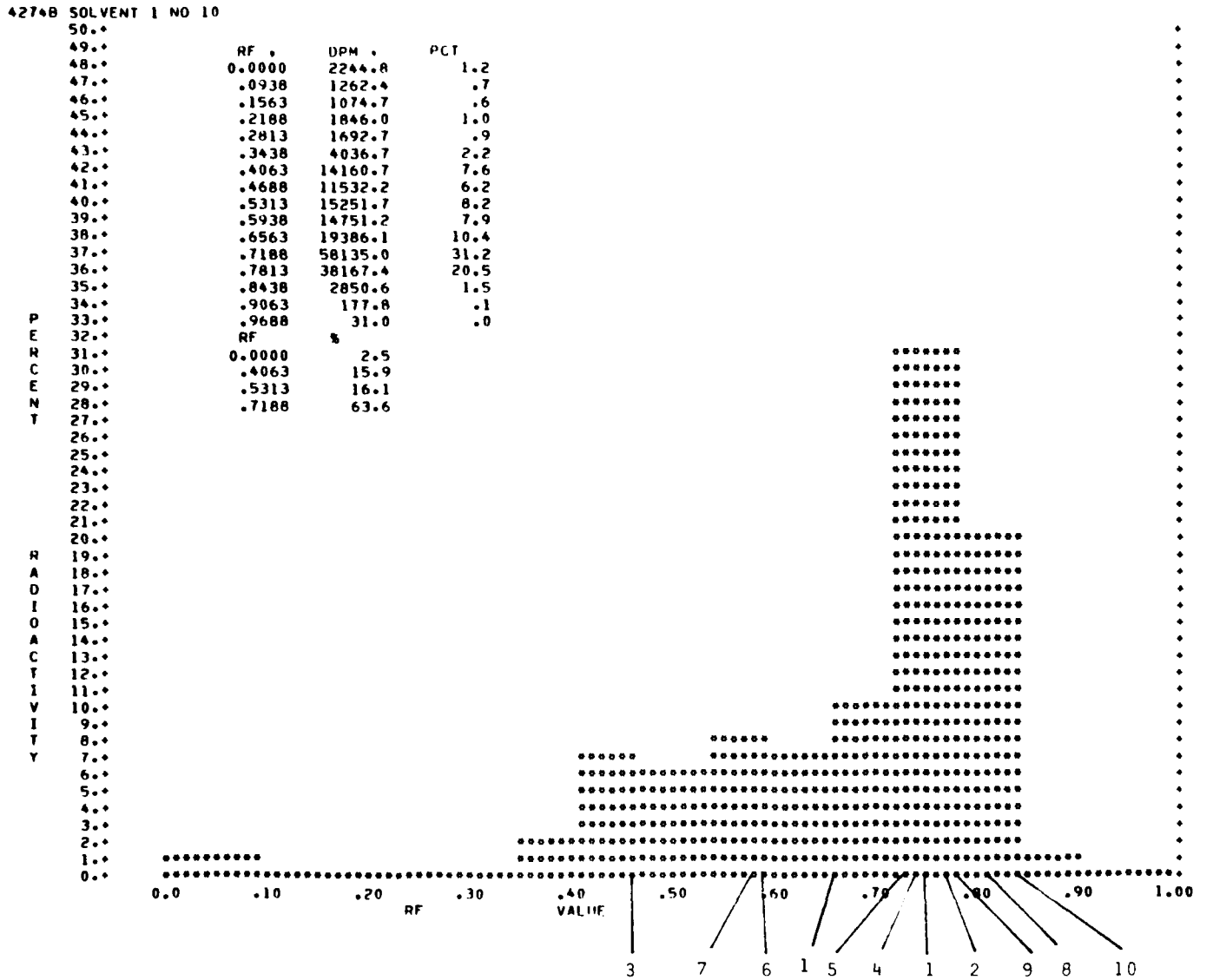

Figure 17-b-I: Oral Treatment, Incubation with B-glucuronidase, Solvent I.

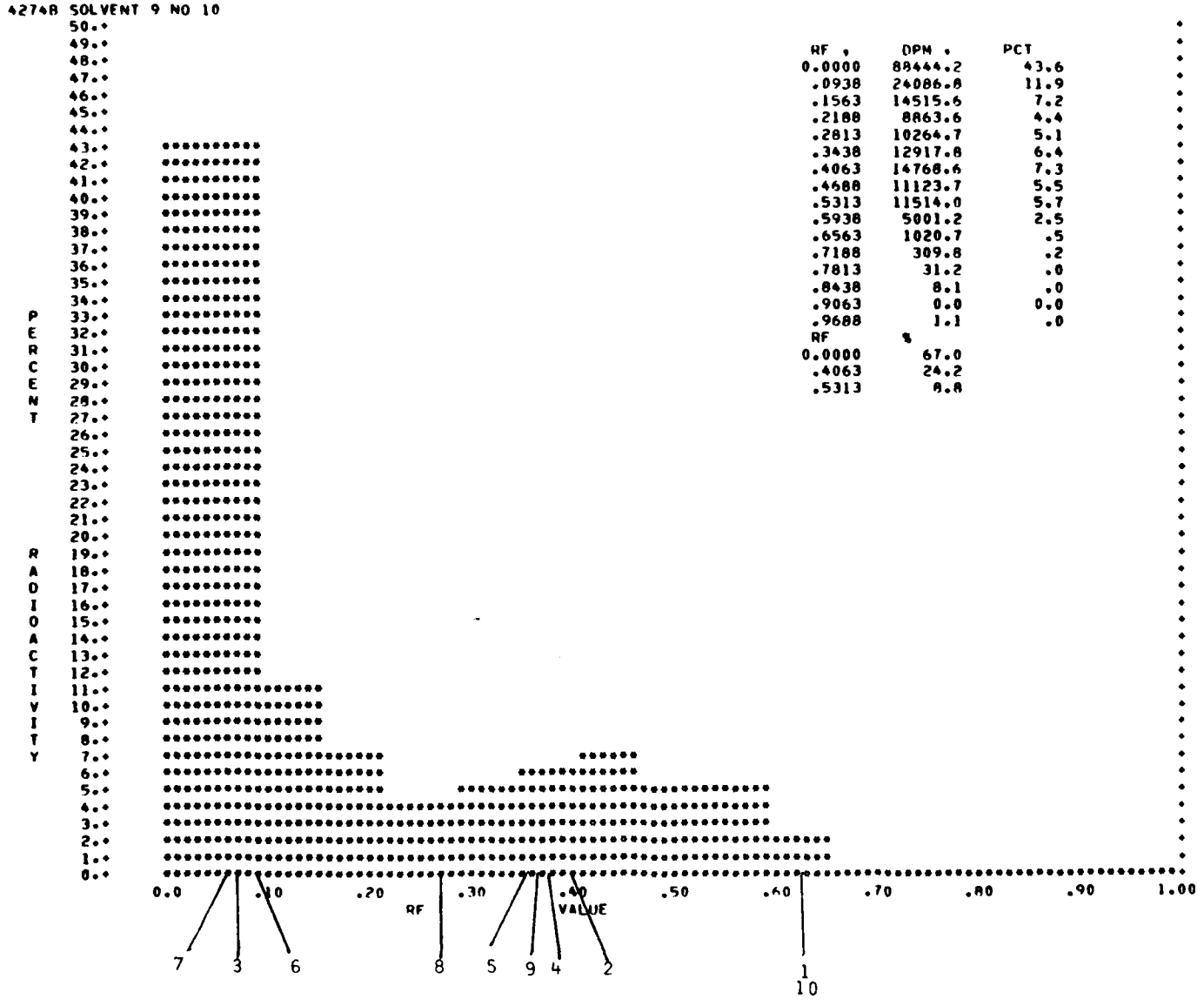

Figure 17-b-IX: Oral Treatment, Incubation with B-glucuronidase, Solvent IX.

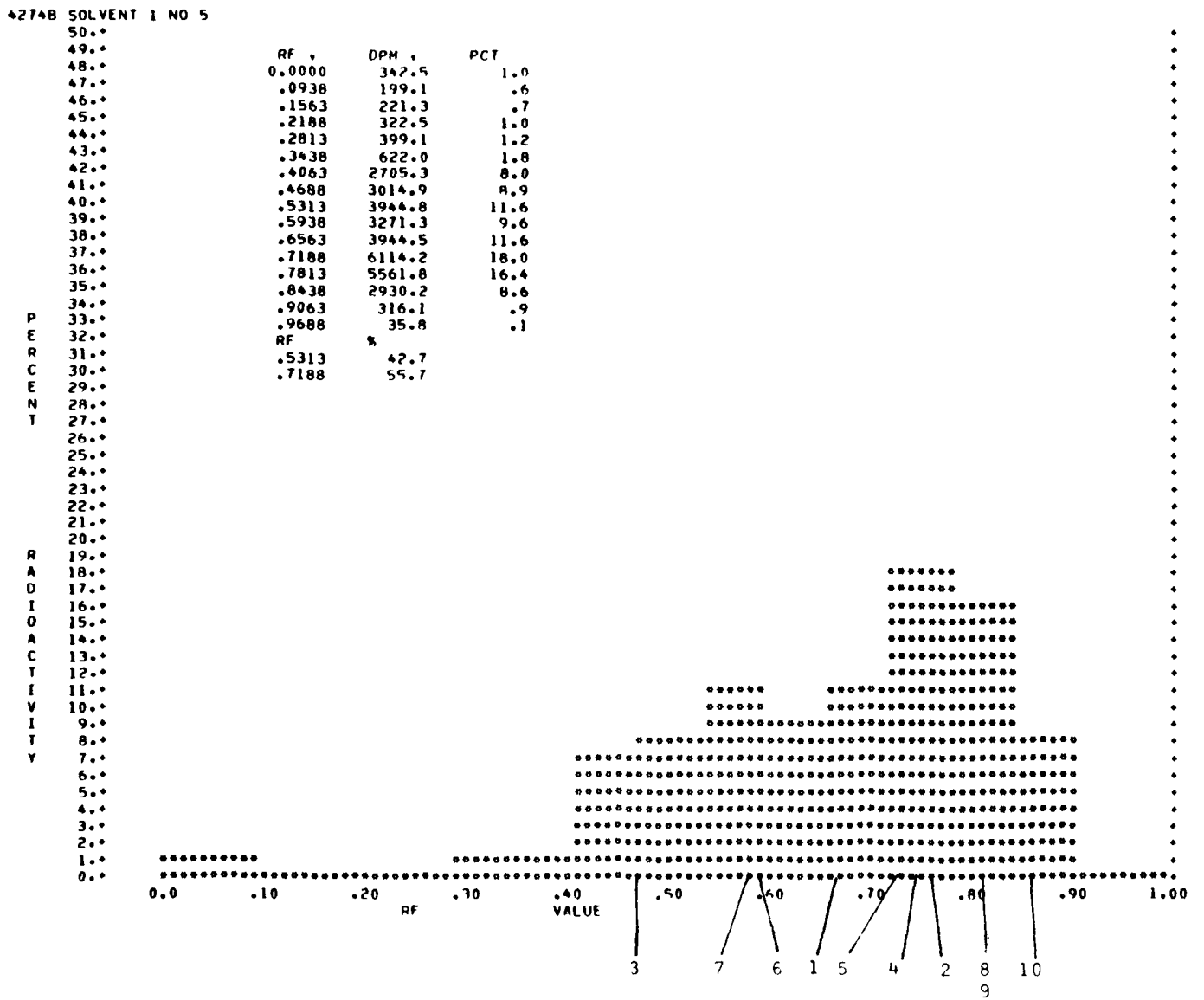

Figure 17-c-I: Dermal Application, Incubation with Water, Solvent I.

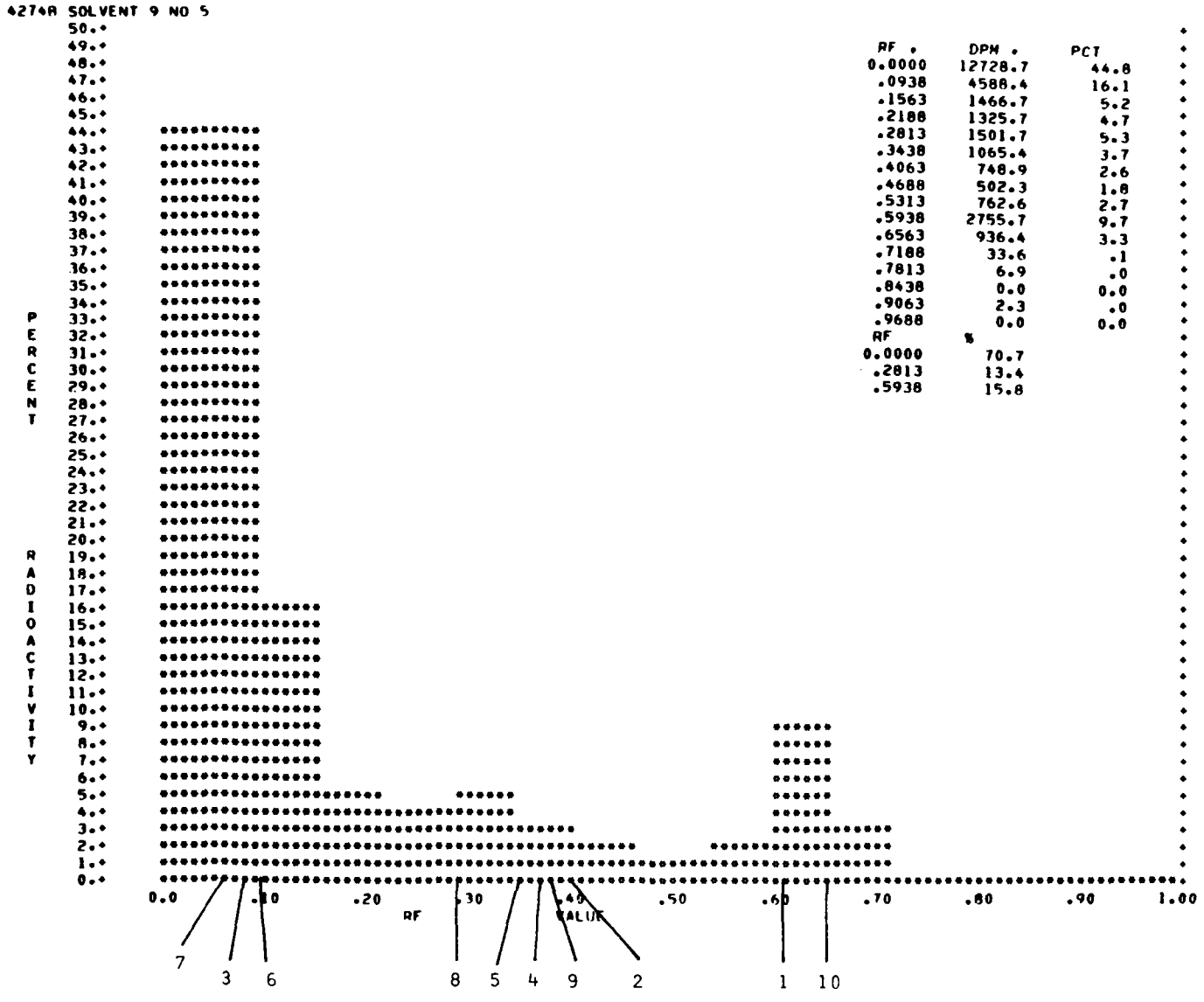

Figure 17-c-IX: Dermal Application, Incubation with Water, Solvent IX.

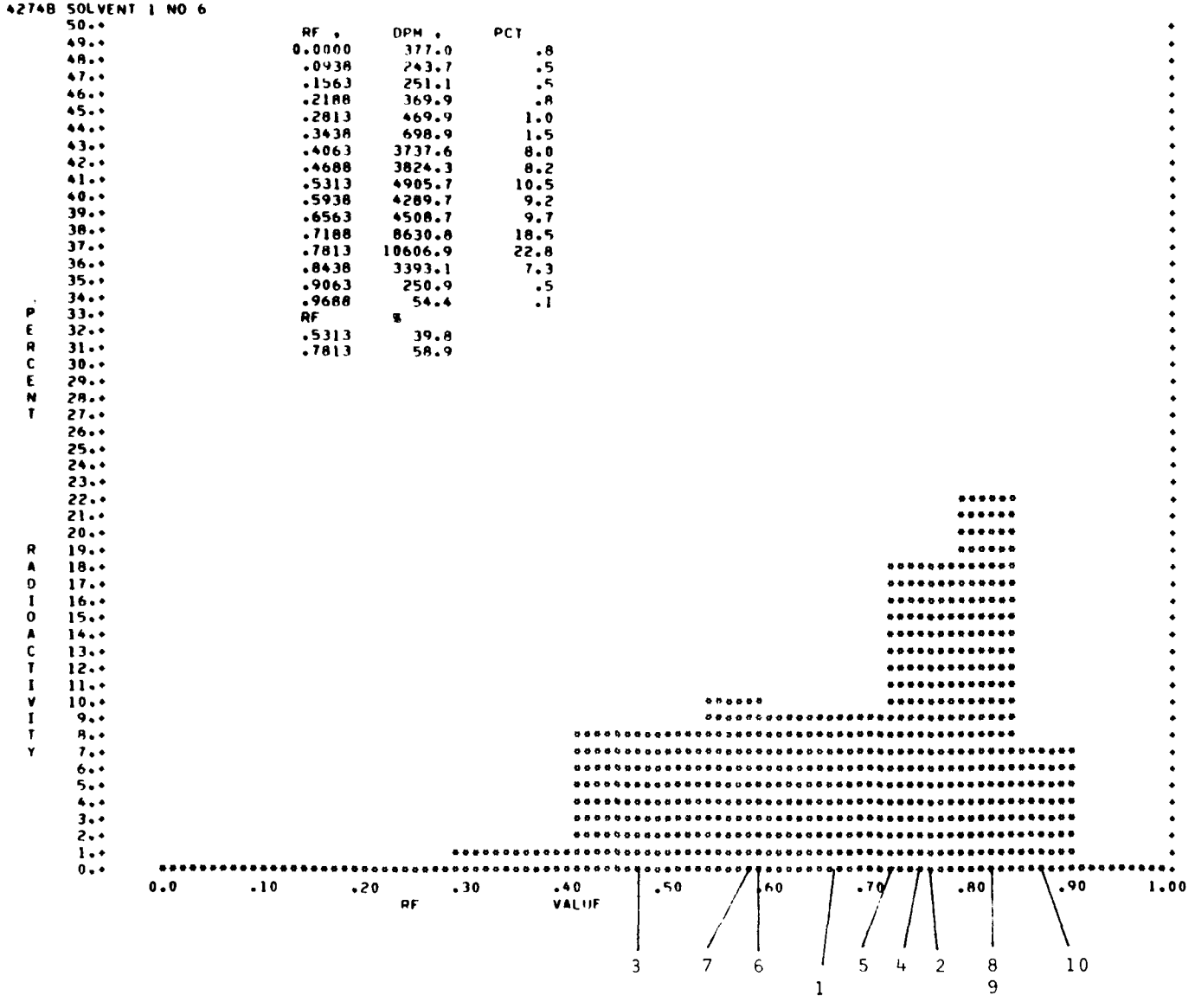

Figure 17-d-I: Dermal Application, Incubation with B-glucuronidase, Solvent I.

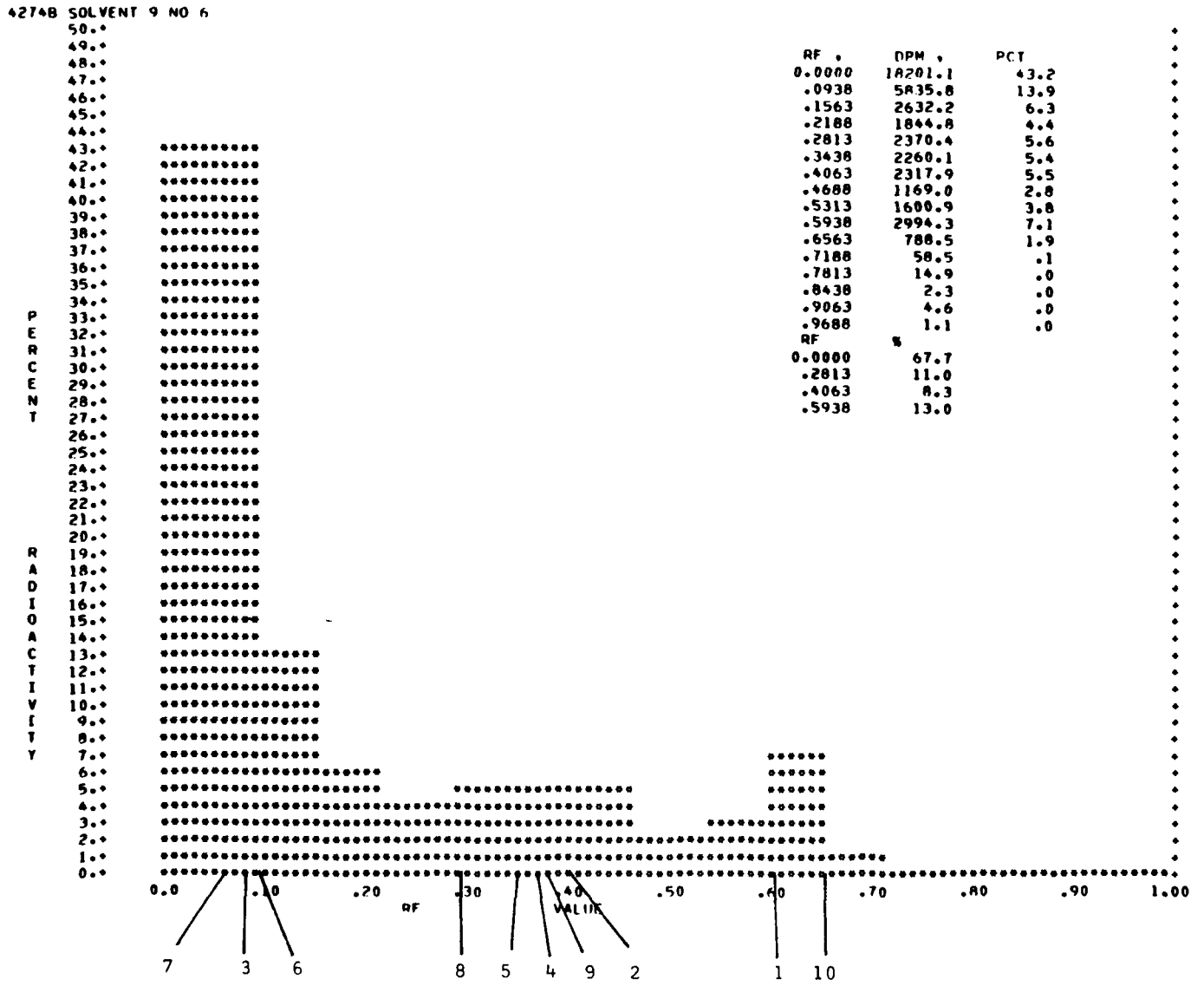

Figure 17-d-IX: Dermal Application, Incubation with B-glucuronidase, Solvent IX.

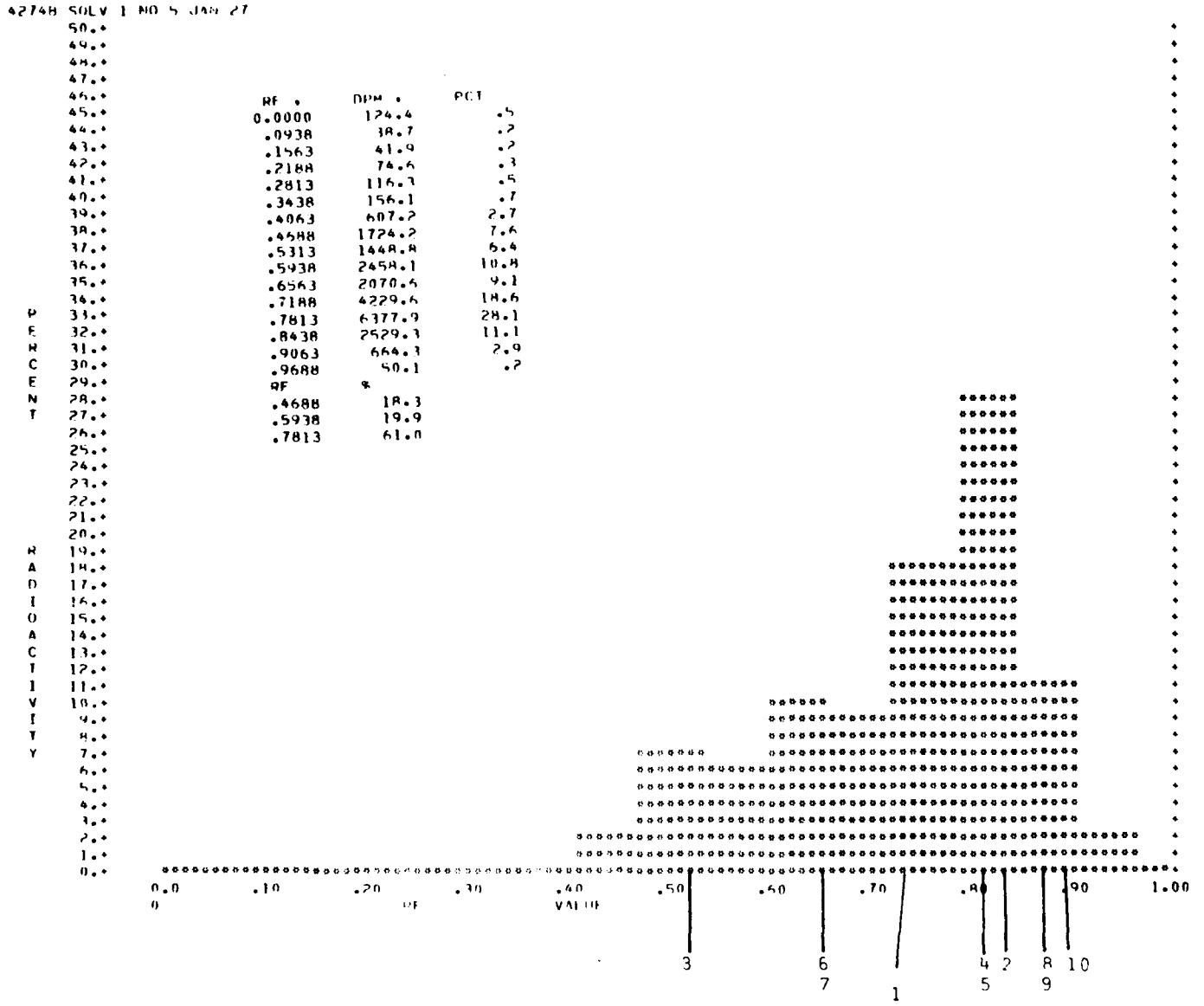

Figure 17-e-I: Oral Treatment, Incubation with Water, Solvent I.

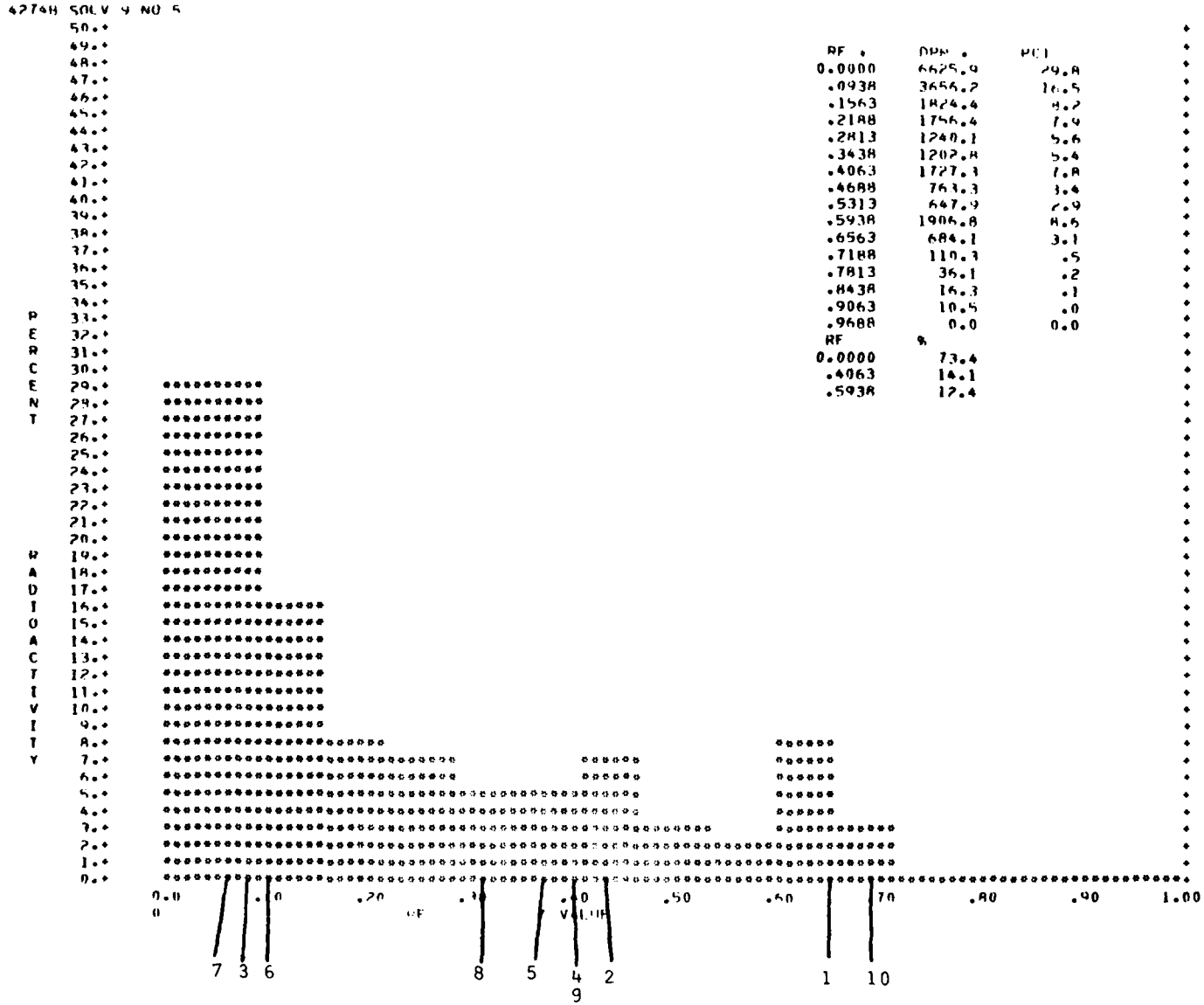

Figure 17-e-IX: Oral Treatment, Incubation with Water, Solvent IX.

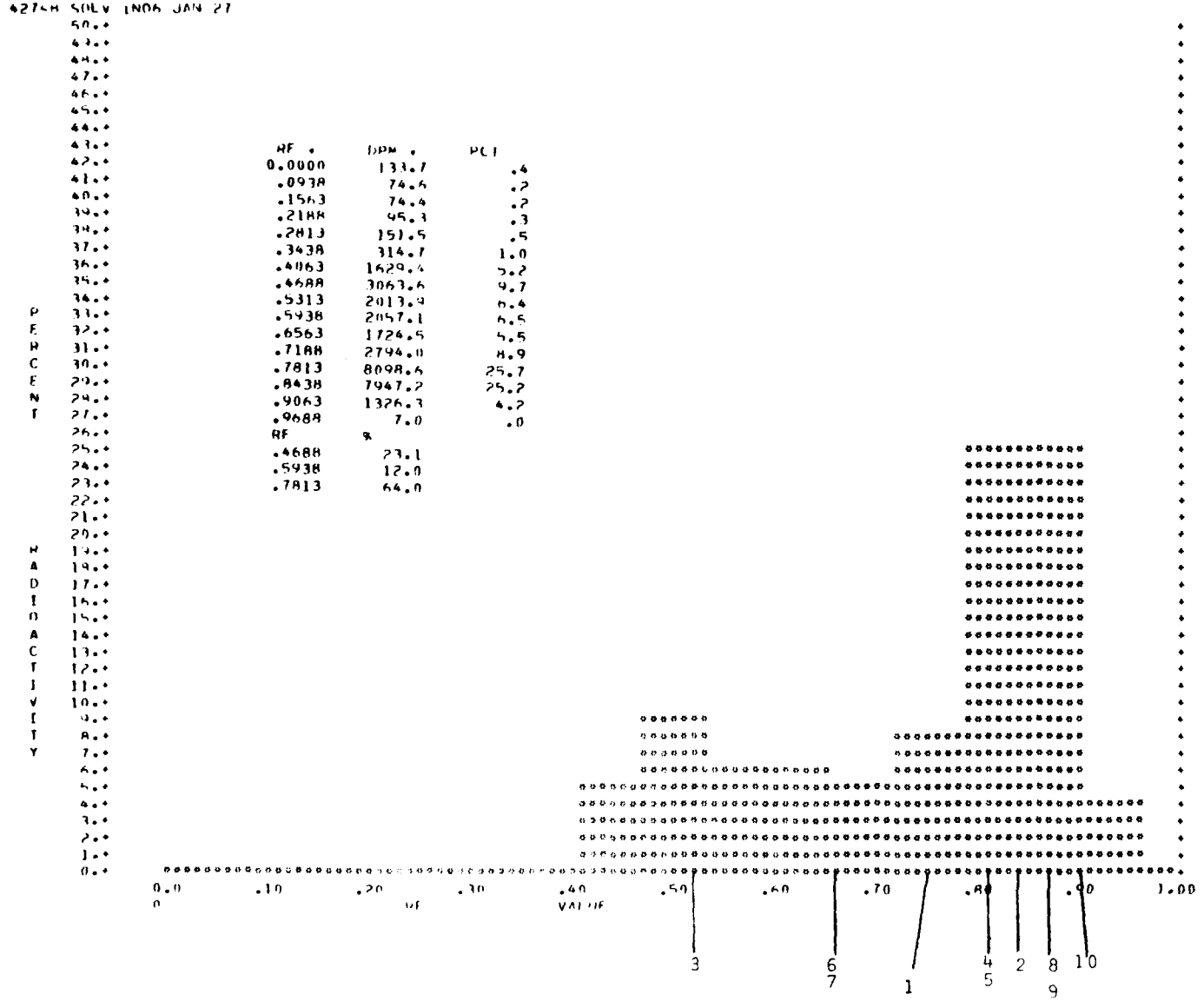

Figure 17-f-I: Oral Treatment, Incubation with B-glucuronidase, Solvent I.

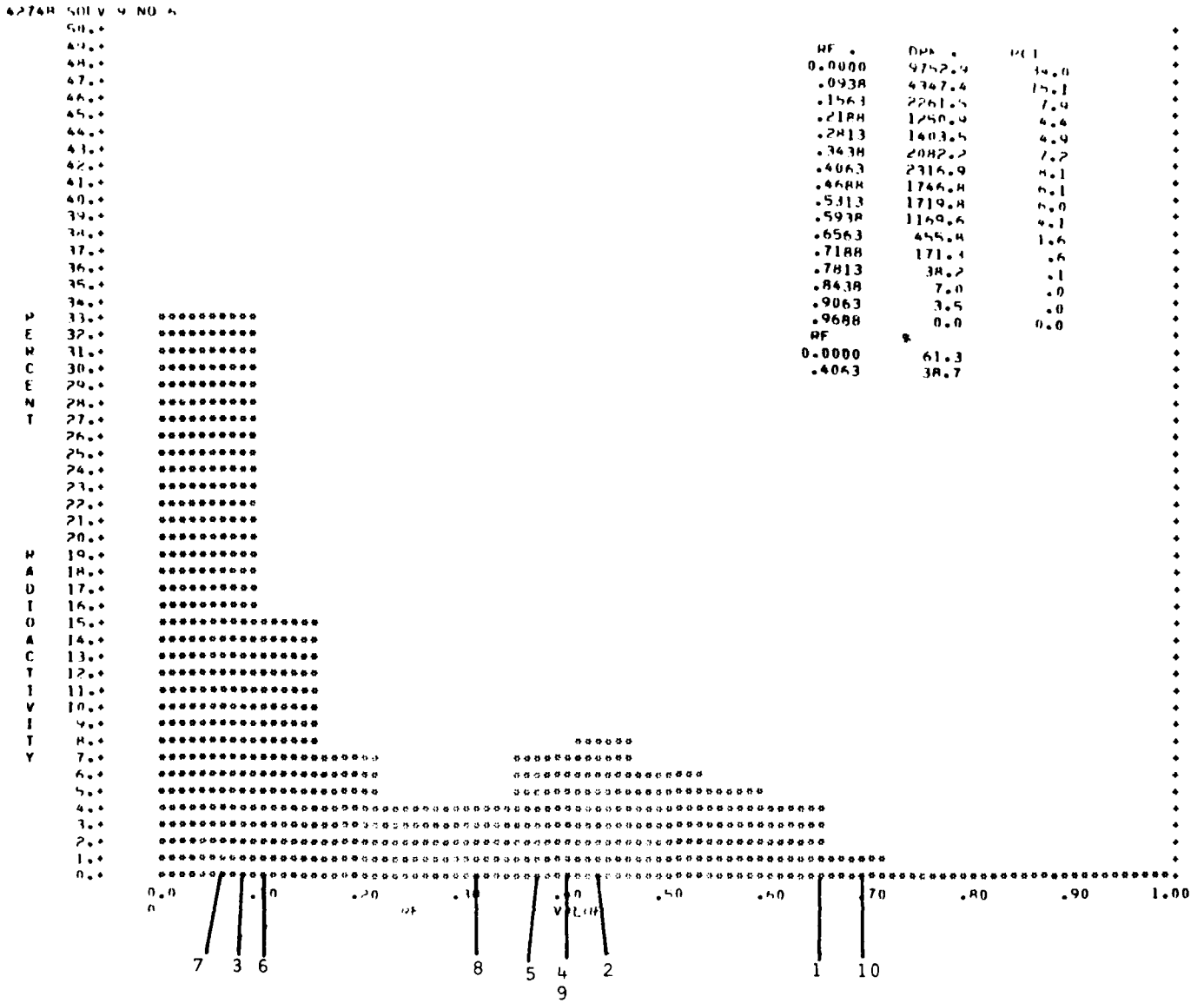

Figure 17-f-IX: Oral Treatment, Incubation with B-glucuronidase, Solvent IX.

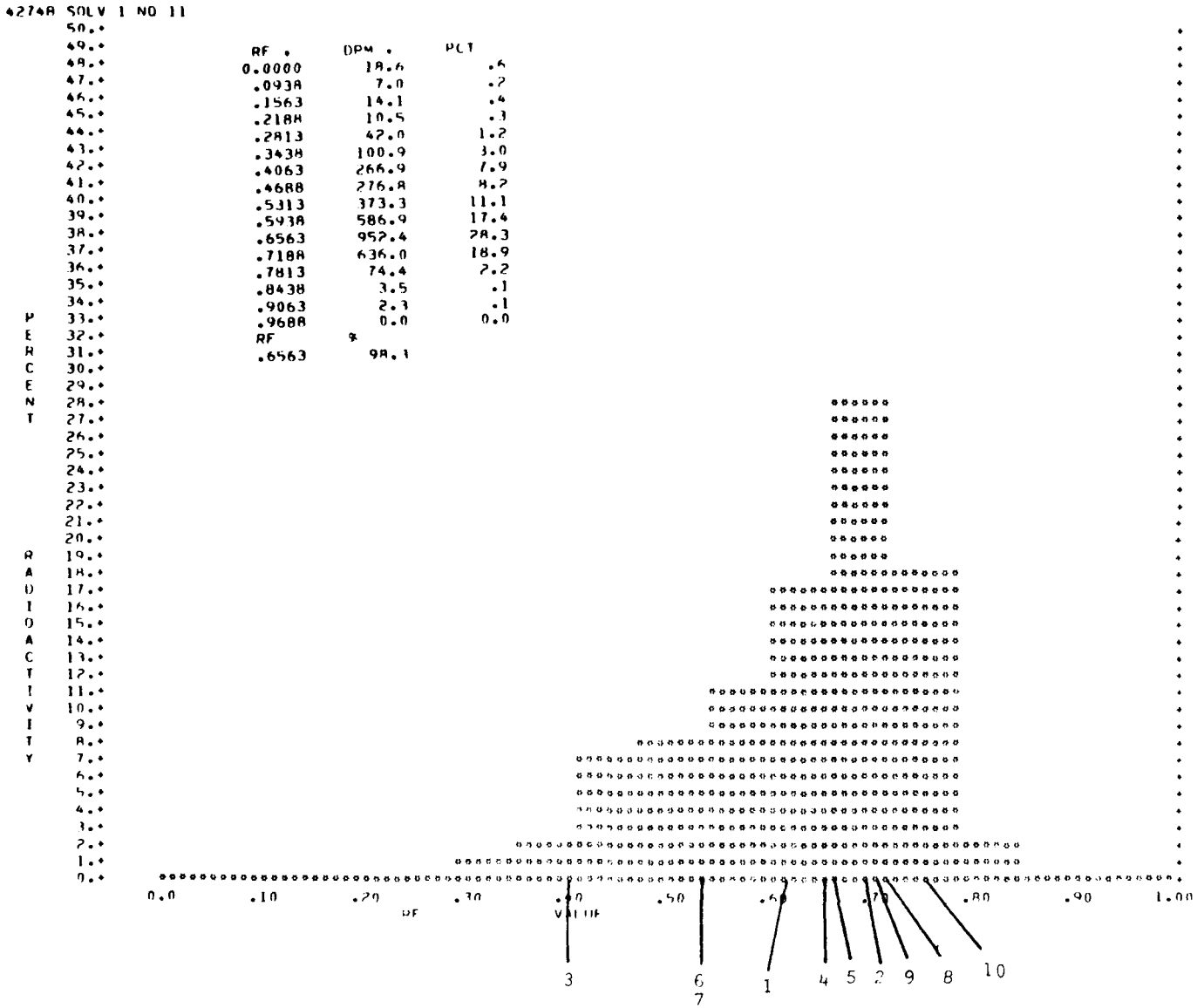

Figure 17-g-I: Dermal Application, Incubation with Water, Solvent I.

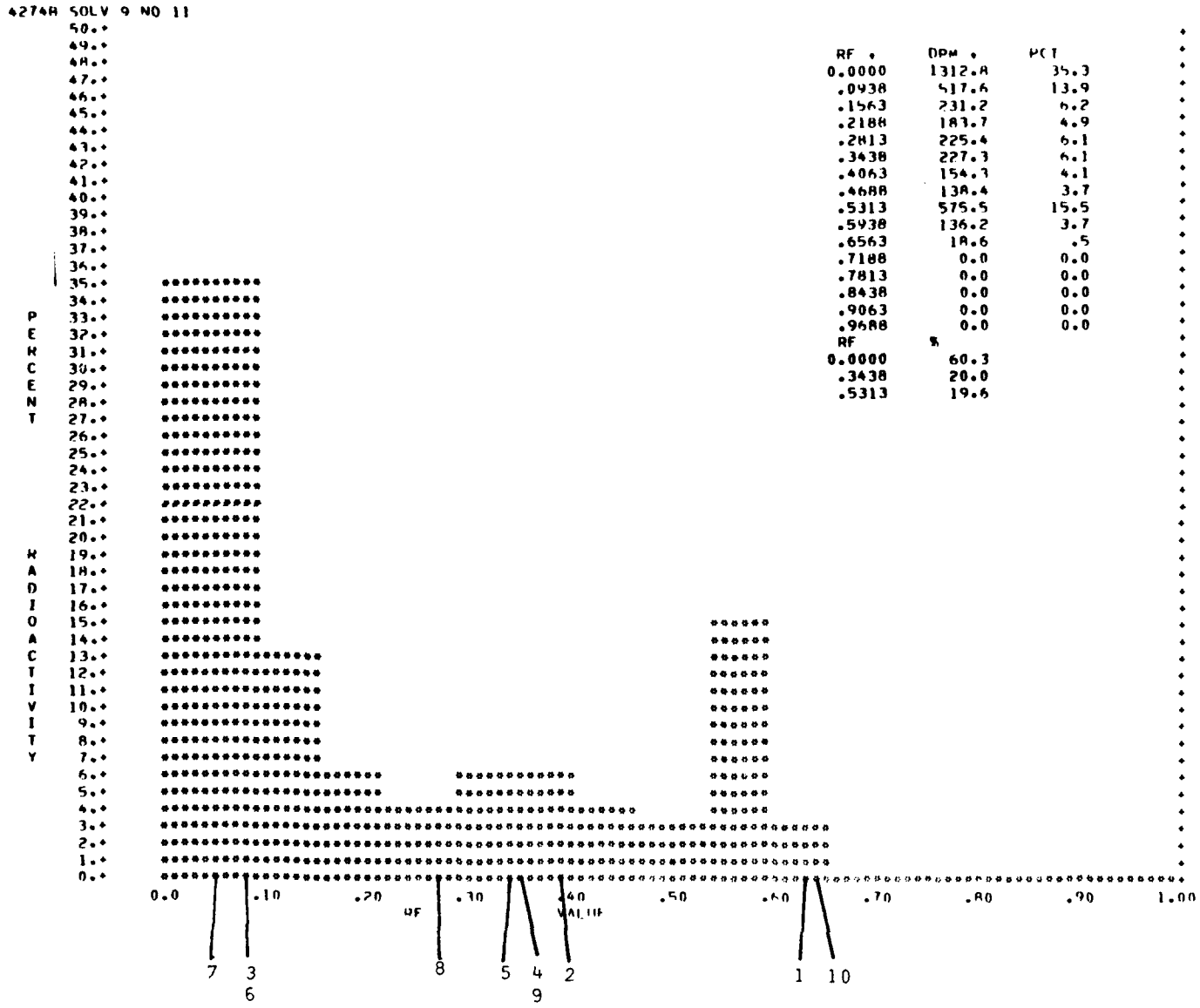

Figure 17-g-IX: Dermal Application, Incubation with Water, Solvent IX.

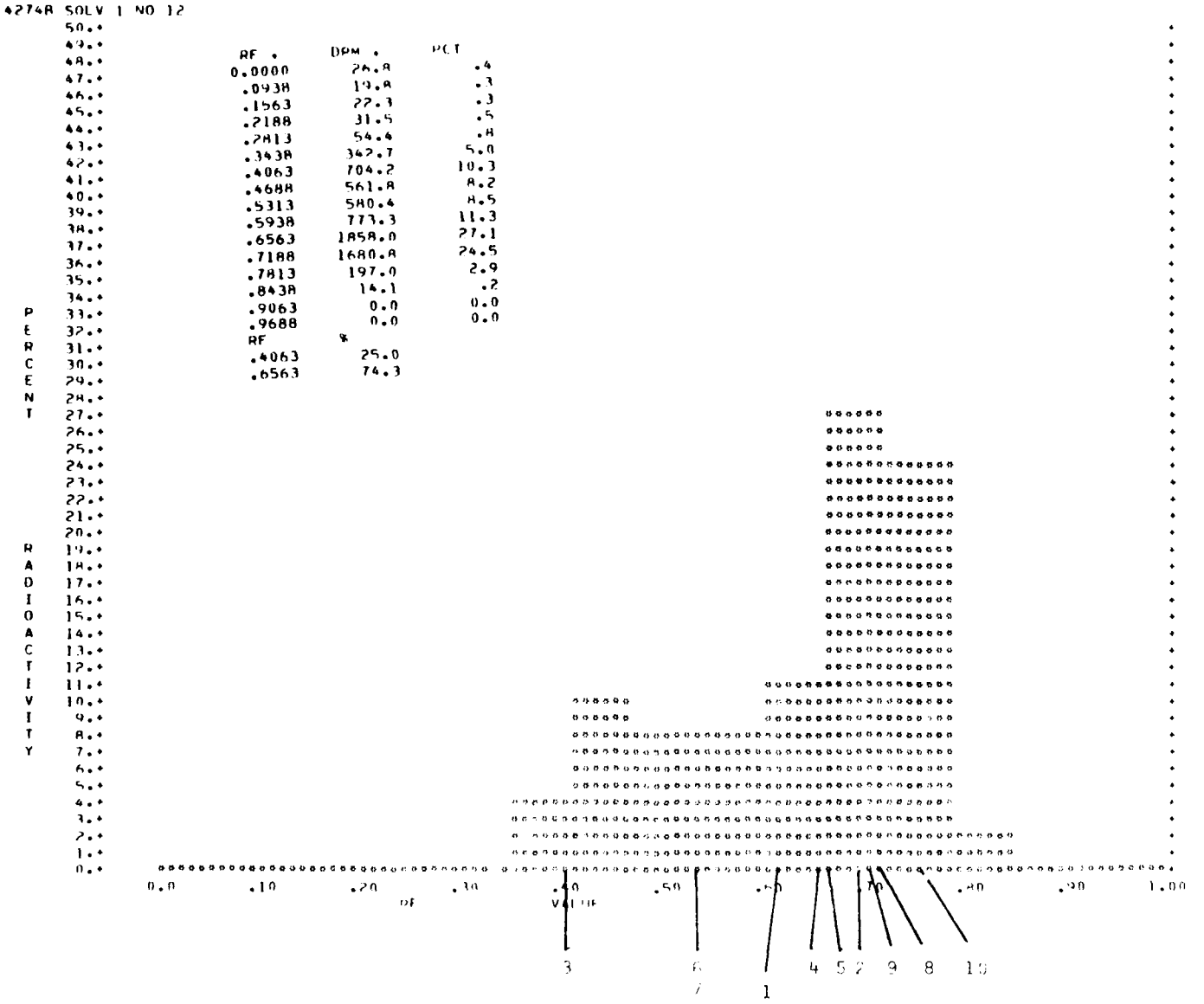

Figure 17-h-I: Dermal Application, Incubation with B-glucuronidase, Solvent I.

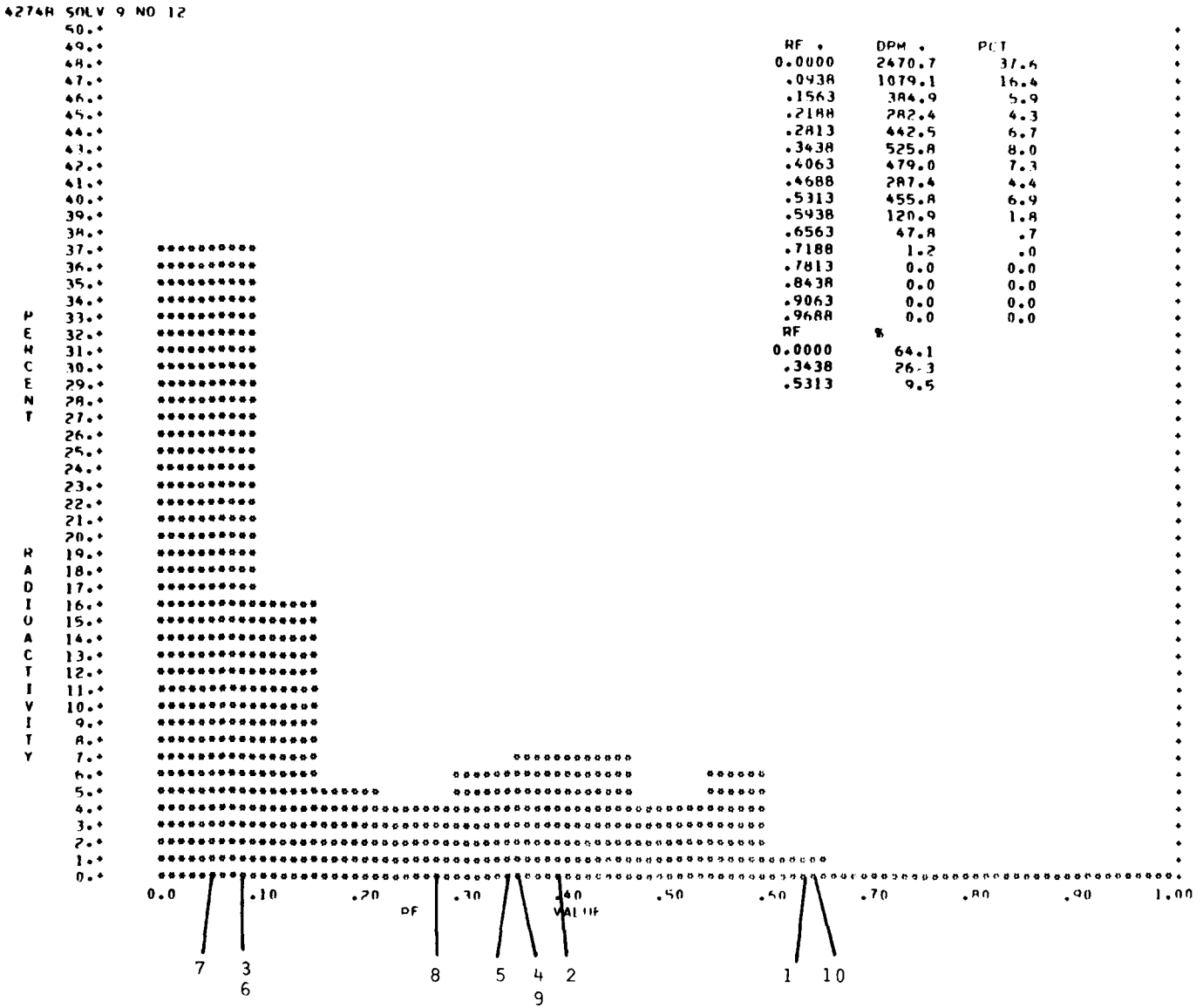

Figure 17-h-IX: Dermal Application, Incubation with β-glucuronidase, Solvent IX.

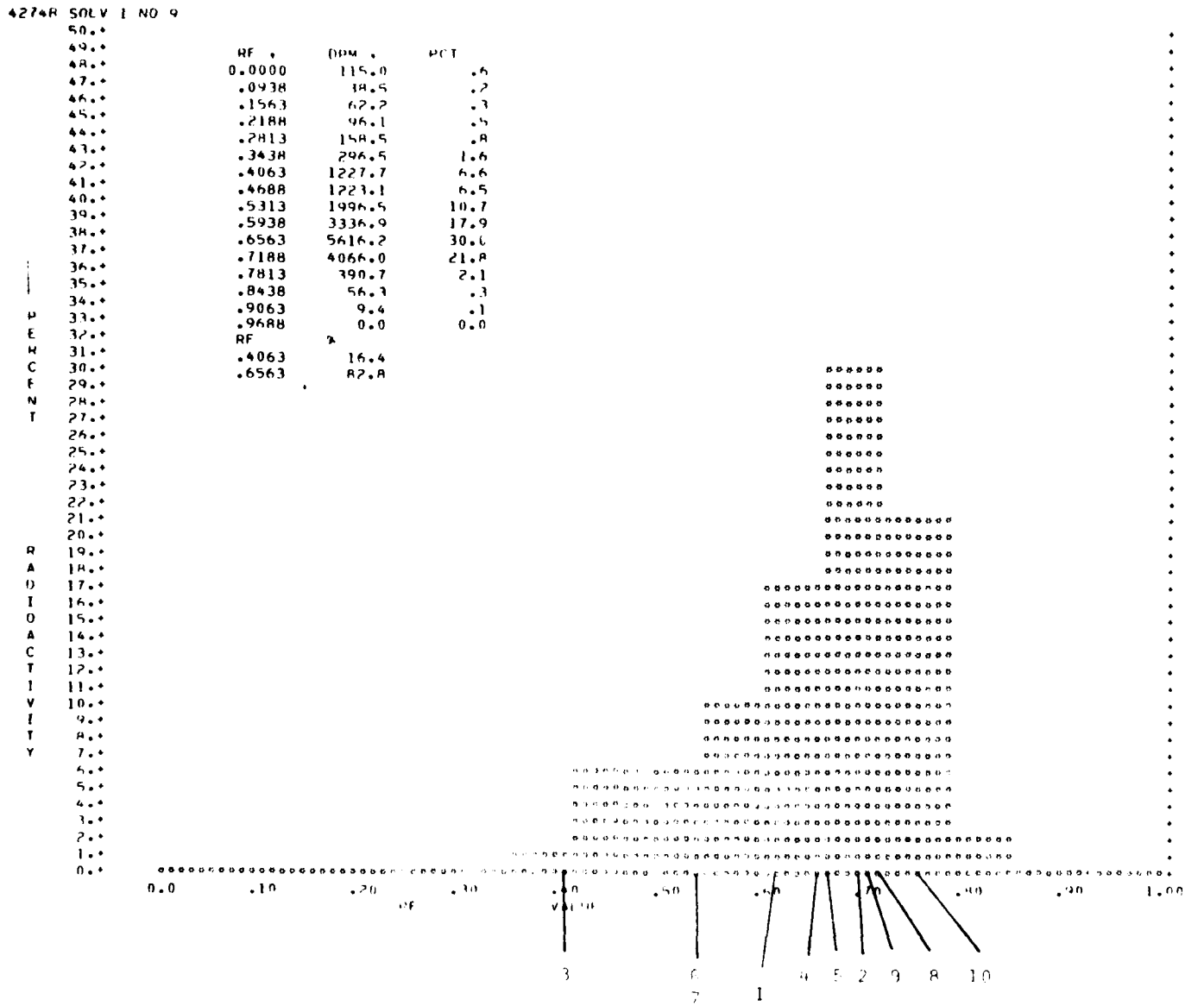

Figure 17-k-1: Oral Treatment, Incubation with Water, Solvent I.

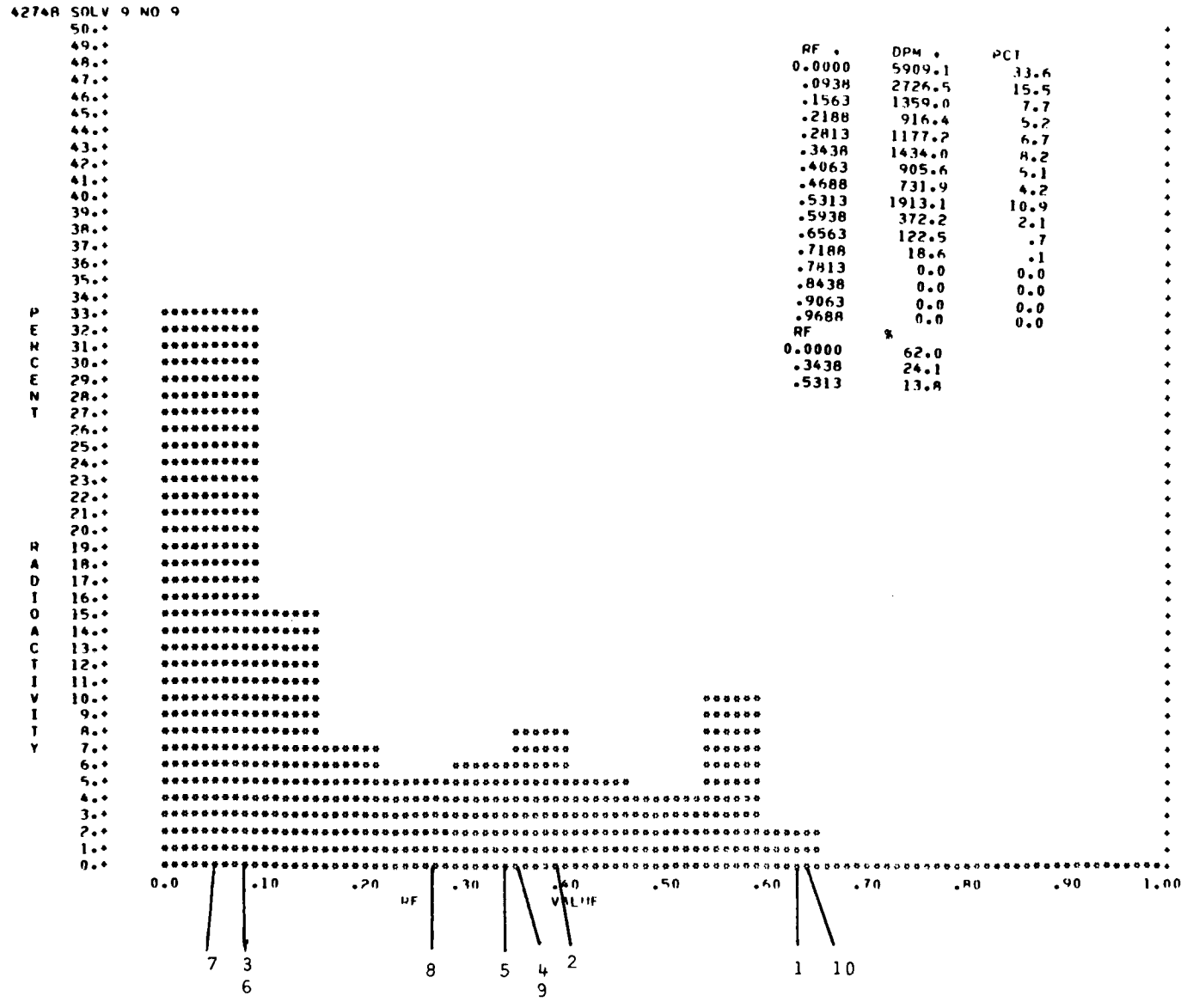

Figure 17-k-IX: Oral Treatment, Incubation with Water, Solvent IX.

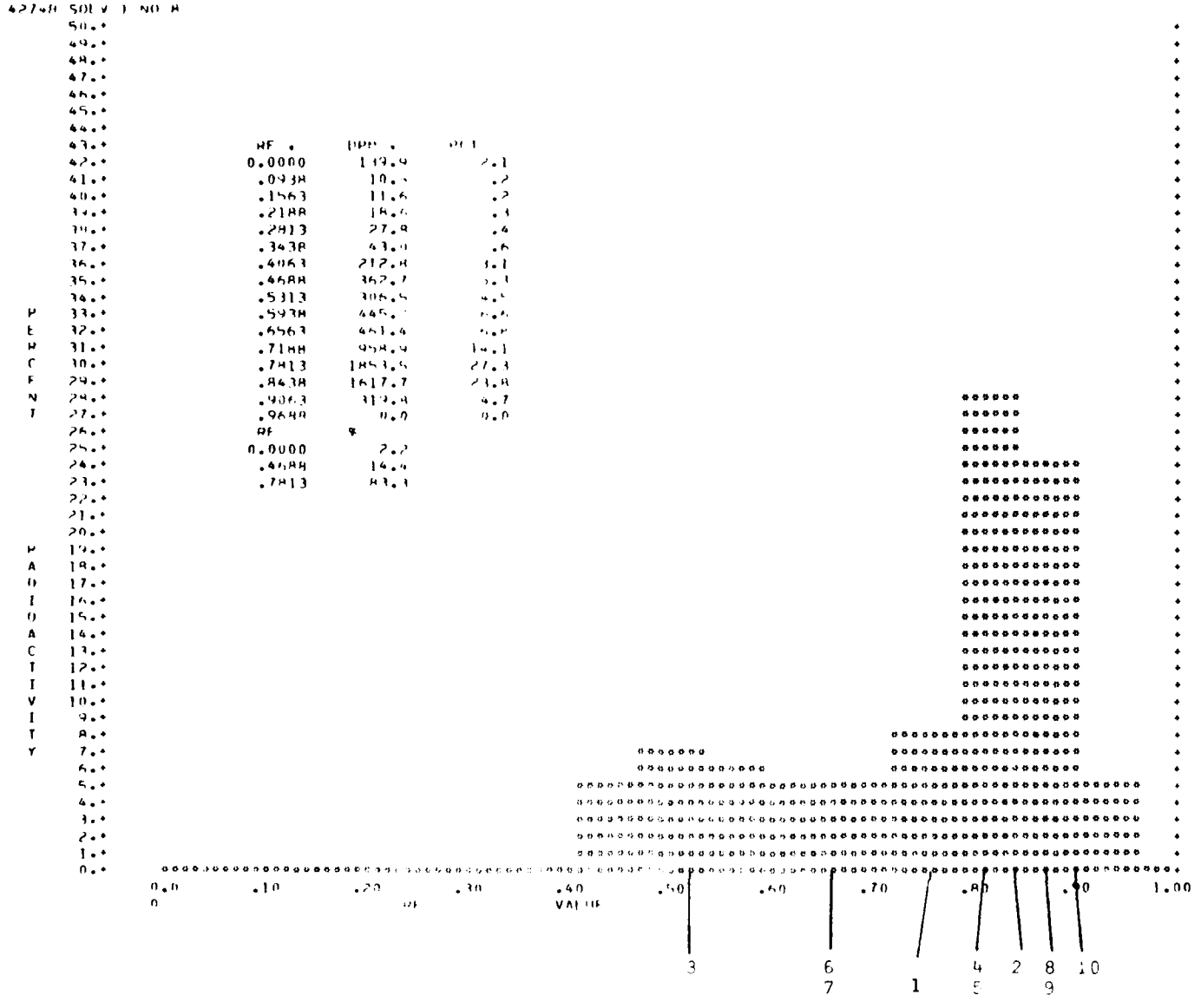

Figure 17-1-I: Dermal Application, Incubation with Water, Solvent I.

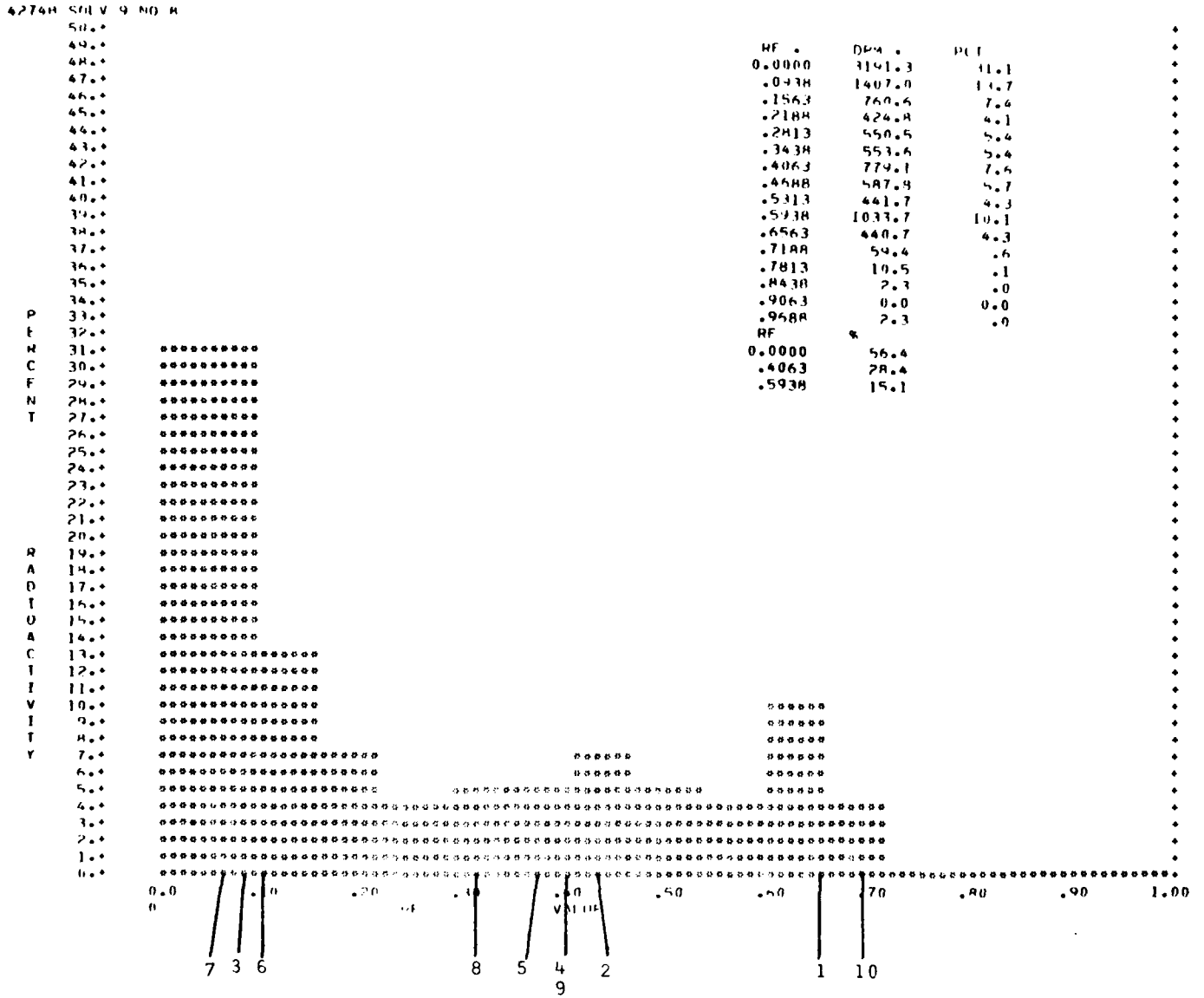

Figure 17-1-IX: Dermal Application, Incubation with Water, Solvent IX.

Figure 18: TLC of Ethyl Acetate-Extractable Products Obtained from 4-Hr Urine of Male Rats Treated Orally or Intratracheally with ^{14}C-TNT. Samples of urine were incubated with acetate buffer and β-glucuronidase before extraction with ethyl acetate. Incubation with acetate buffer and water served as control. Reference standards are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzylalcohol
3. Trinitrobenzoic Acid
4. 4-Amino-2,6-Dinitrotoluene
5. 2-Amino-4,6-Dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2,6'-Tetranitro-4,4'-azoxytoluene

Figure 18 follows

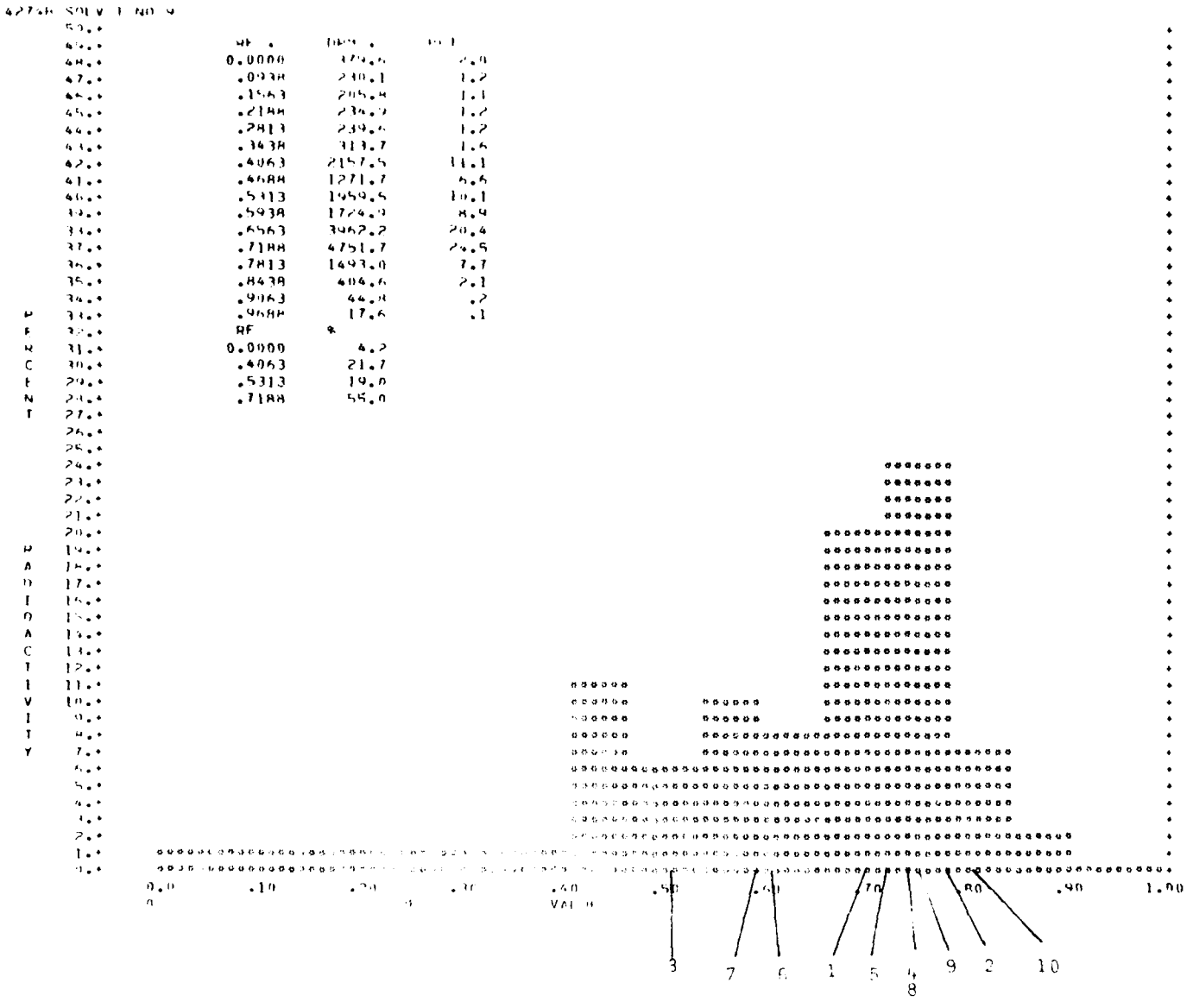

Figure 18-a-I: Oral Treatment, Incubation with Water, Solvent 1

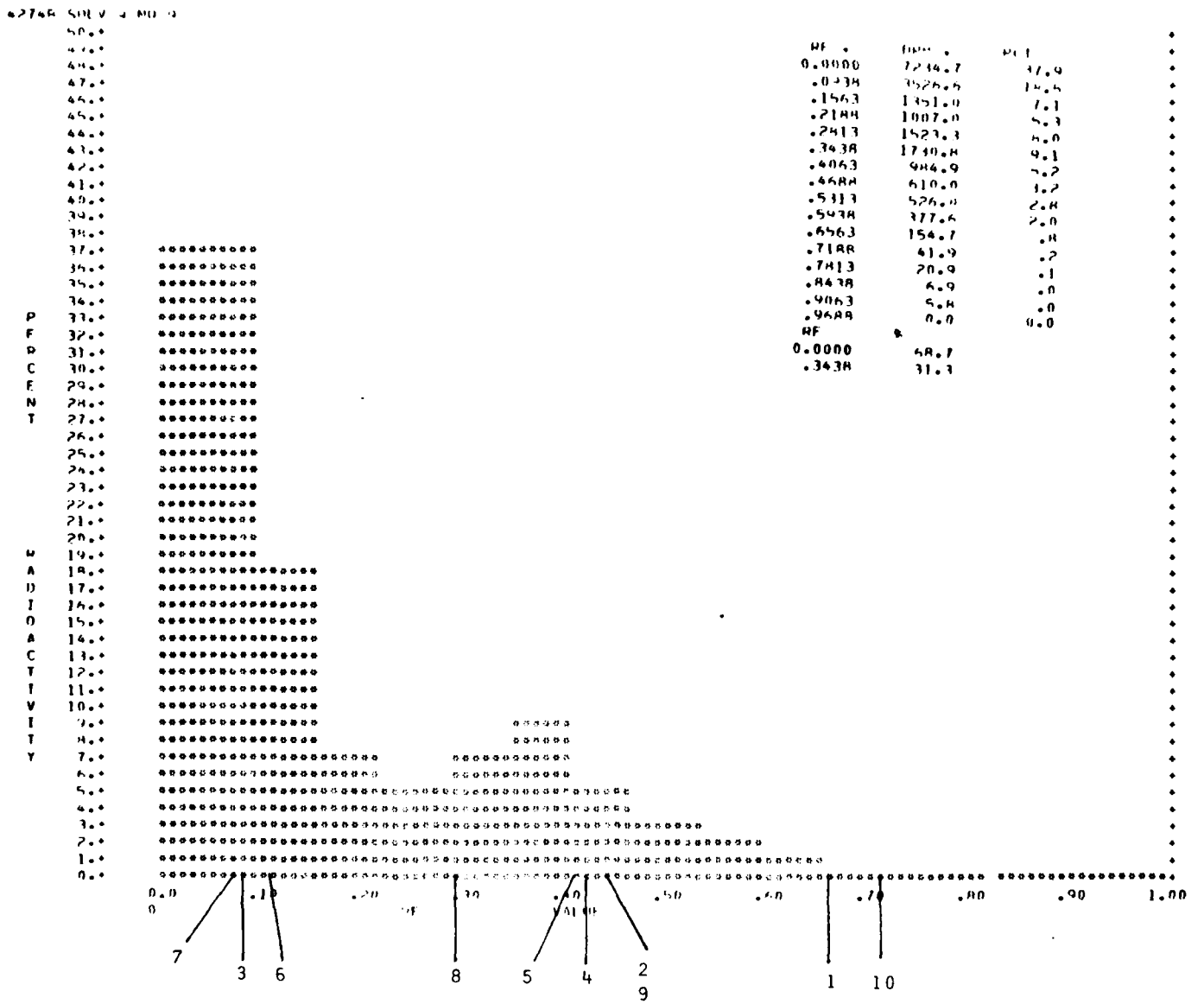

Figure 18-a-IX: Oral Treatment, Incubation with Water, Solvent IX.

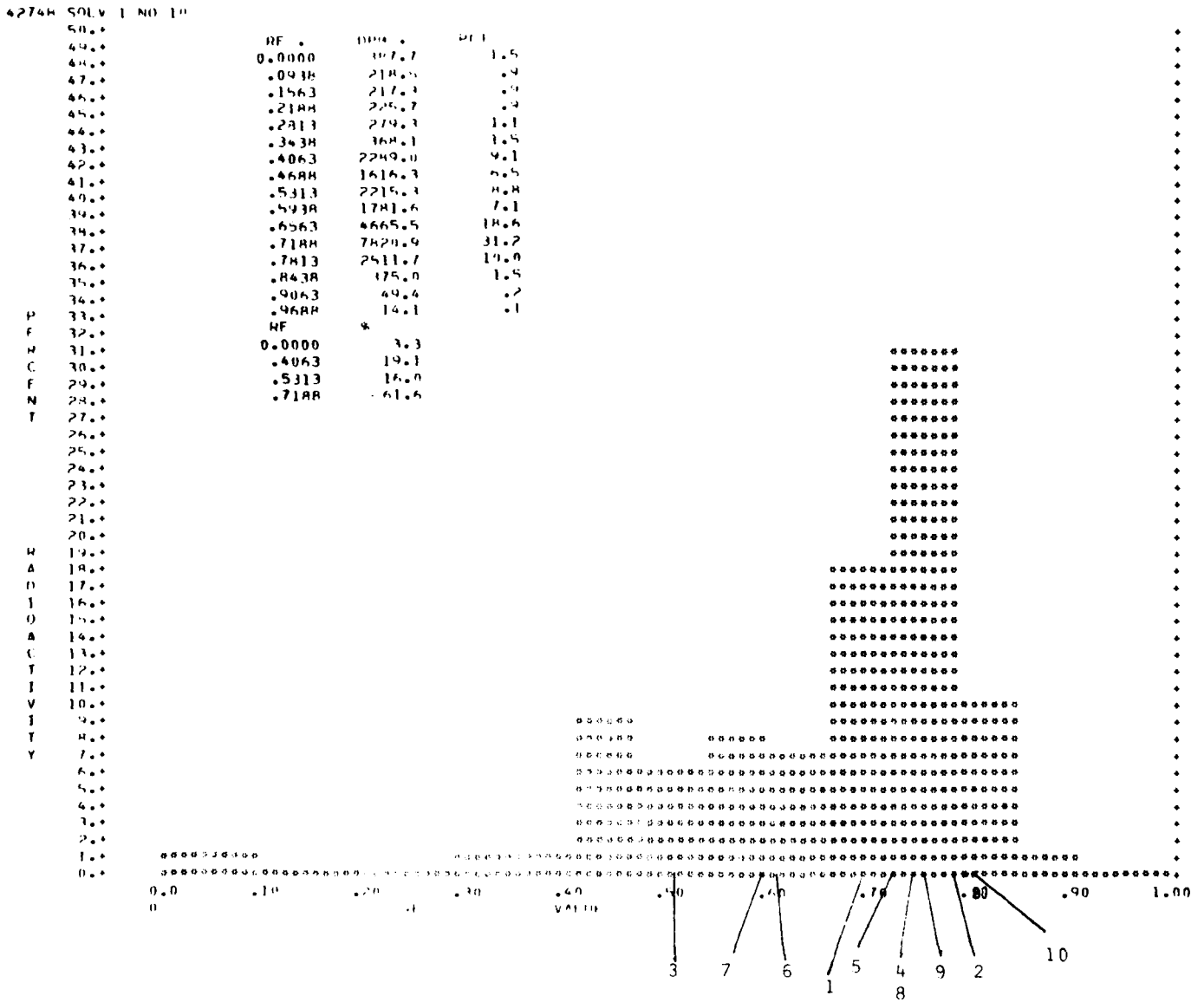

Figure 18-b-I: Oral Treatment, Incubation with B-glucuronidase, Solvent I

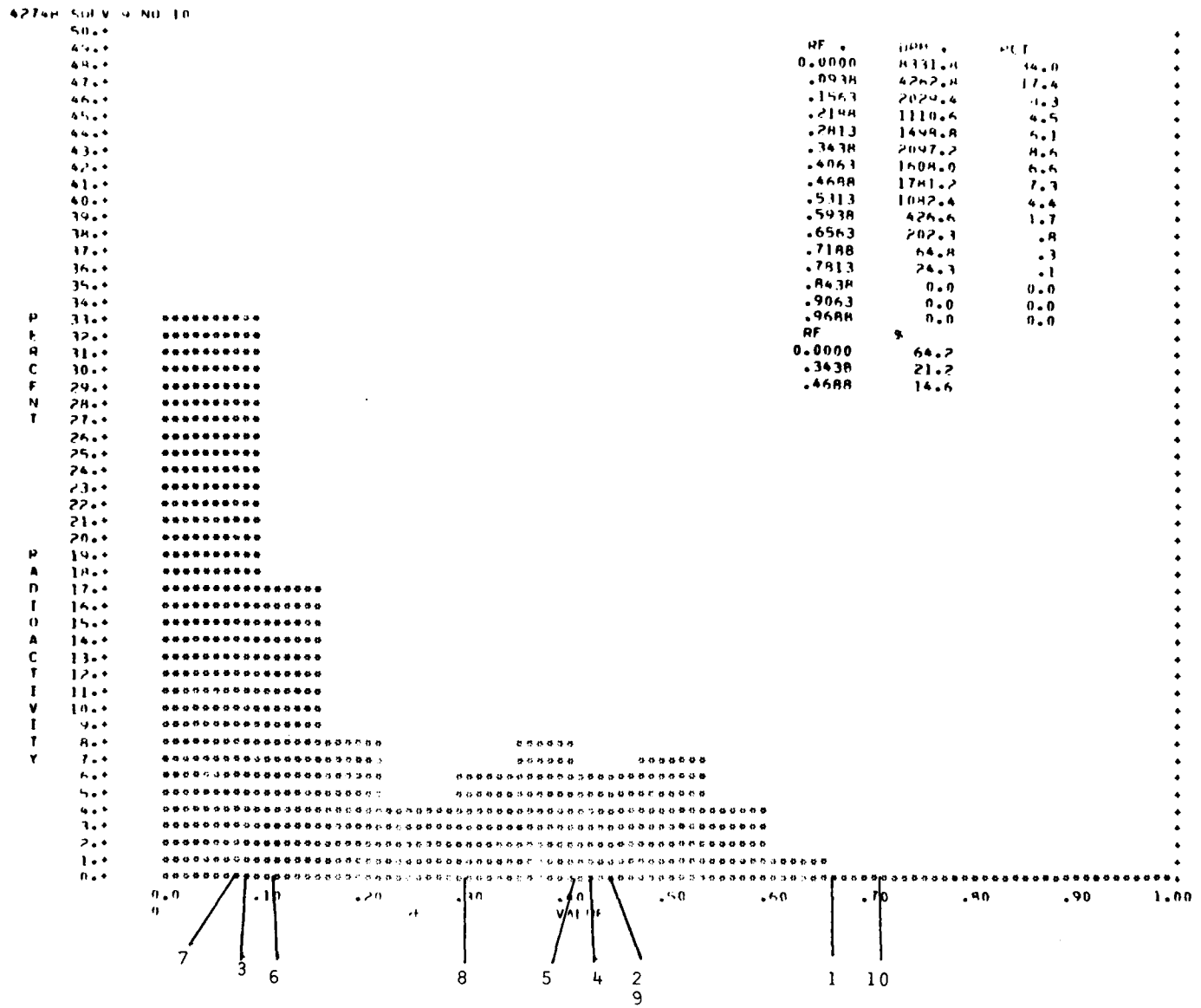

Figure 18-b-IX: Oral Treatment, Incubation with B-glucuronidase, Solvent IX.

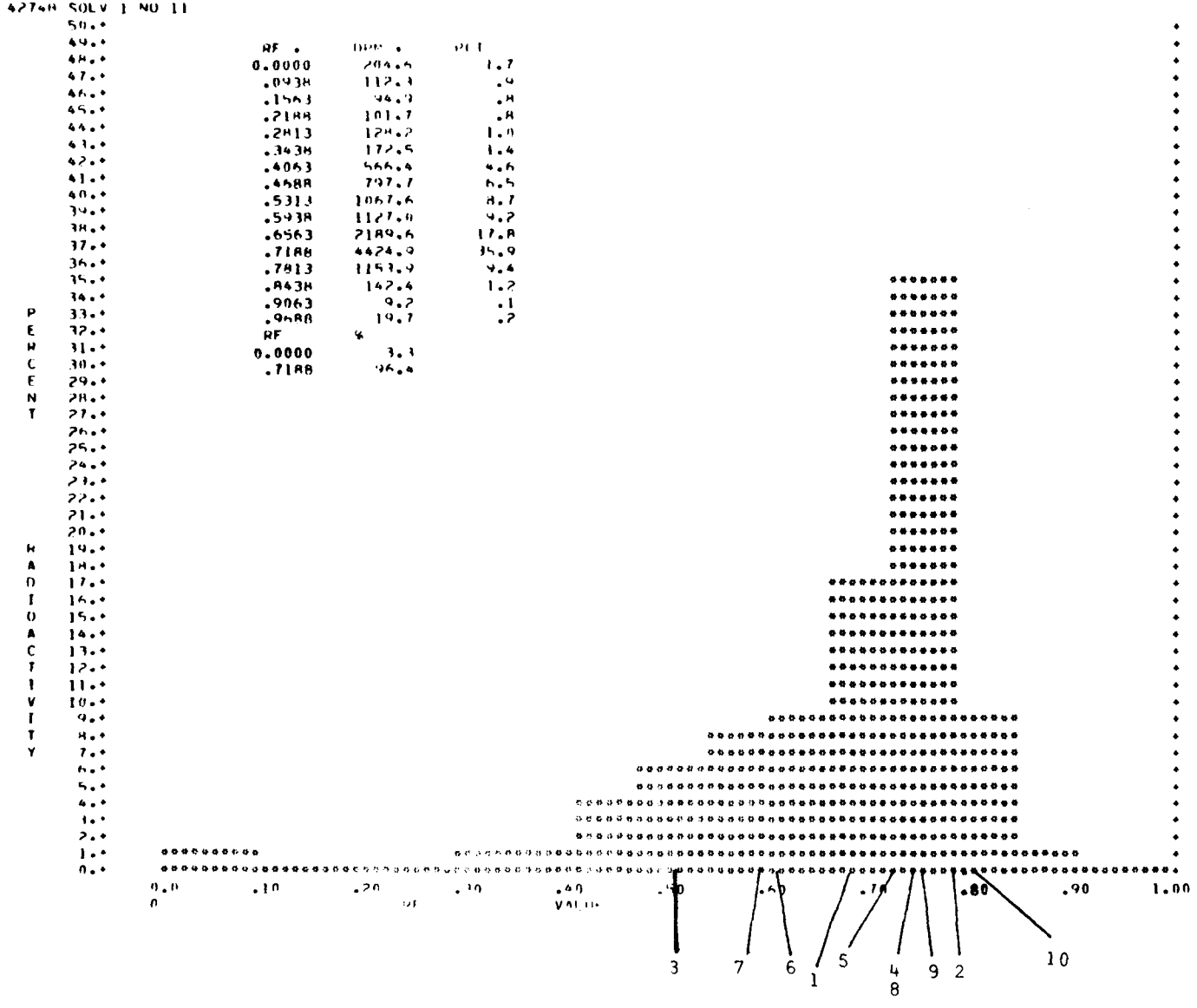

Figure 18-c-I: Intratracheal Instillation, Incubation with Water, Solvent I.

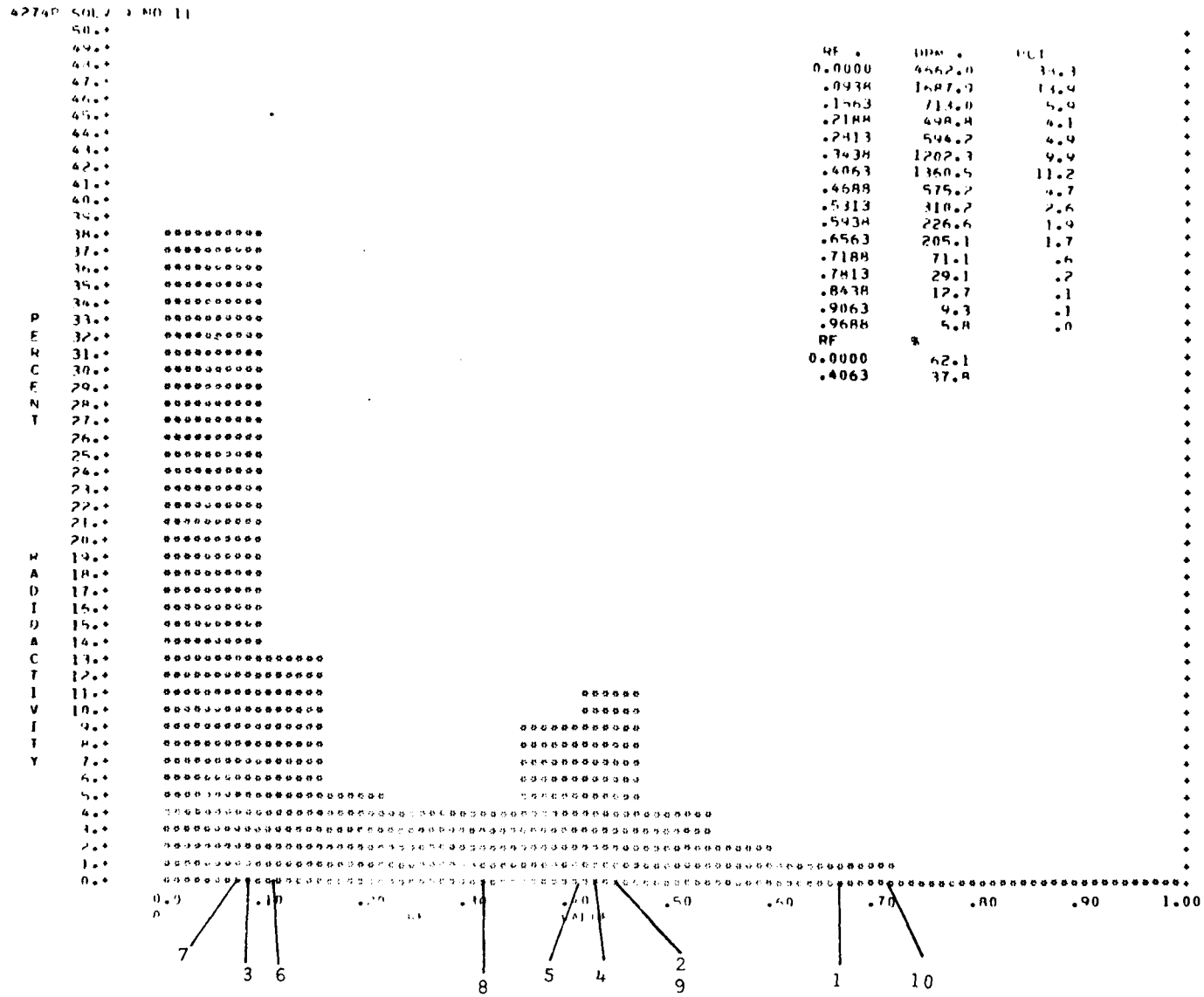

Figure 18-c-IX: Intratracheal Instillation, Incubation with Water, Solvent IX.

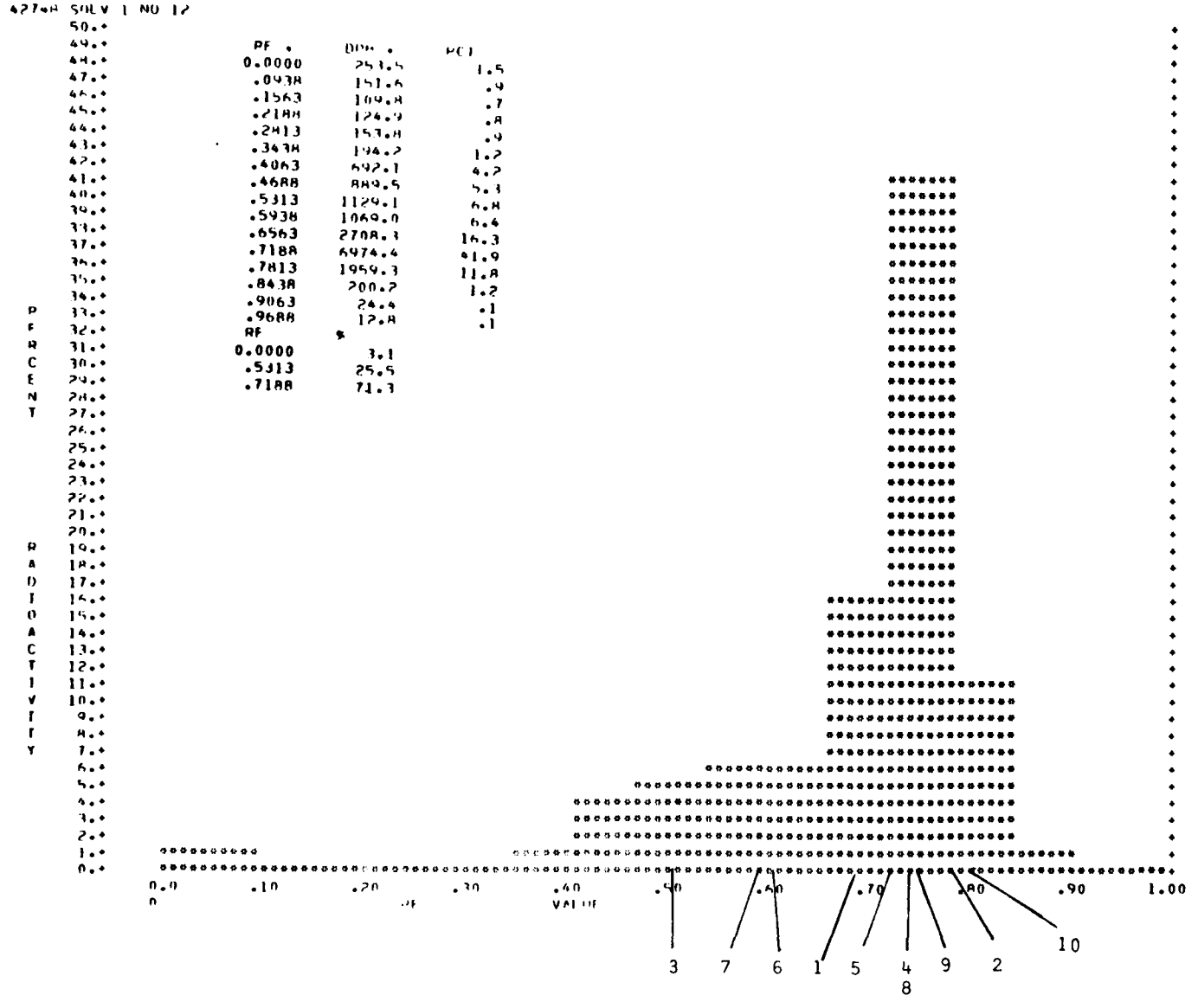

Figure 18-d-I: Intratracheal Instillation, Incubation with B-glucuronidase, Solvent I.

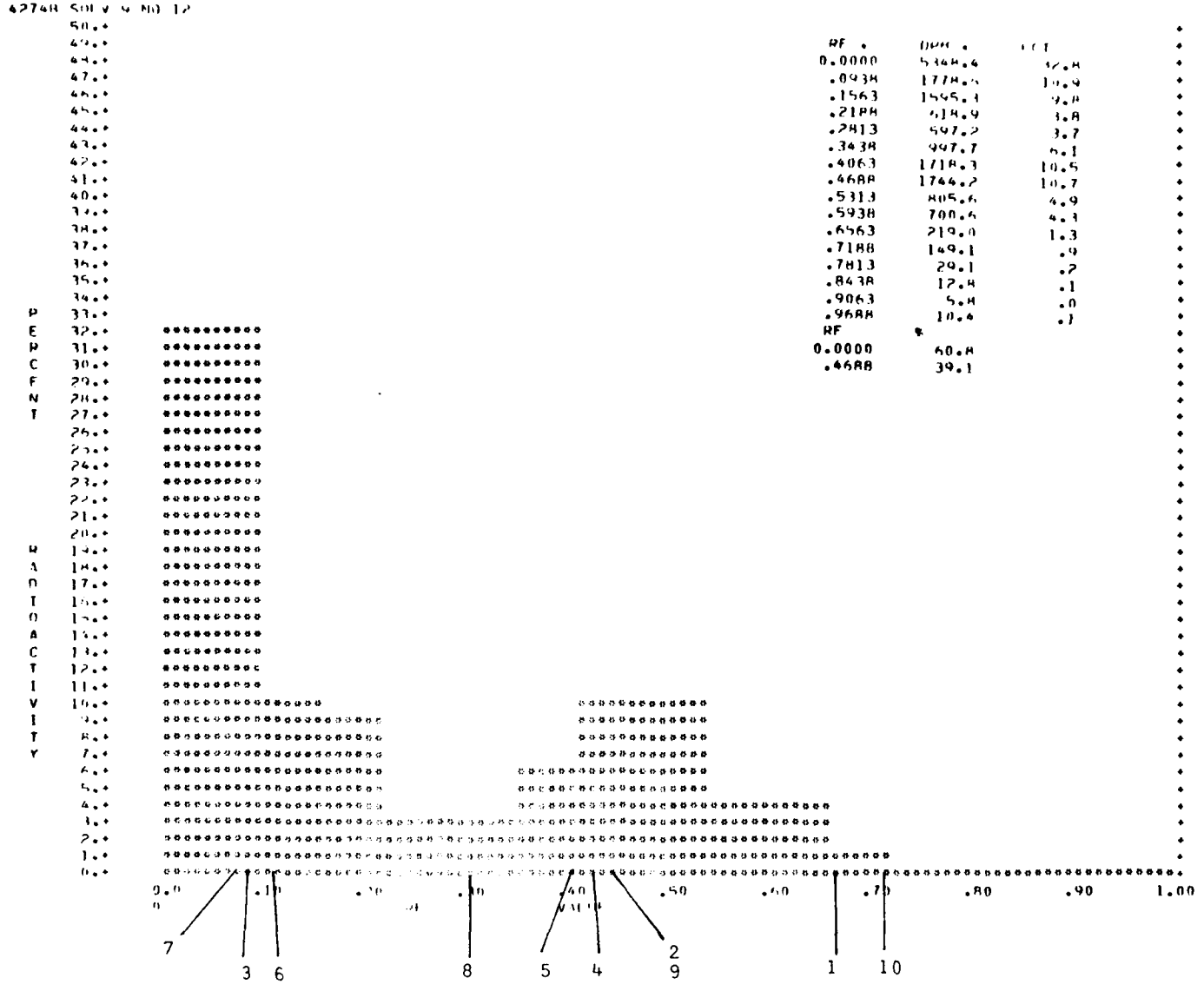

Figure 18-d-IX: Intratracheal Instillation, Incubation with B-glucuronidase, Solvent IX.

Figure 19: TLC of Ethyl Acetate-Extractable Products Obtained from 4-Hr Urine of Female Rats Treated Orally or Intratracheally with ^{14}C-TNT. Samples of urine were incubated with acetate buffer and β-glucuronidase before extraction with ethyl acetate. Incubation with acetate buffer and water served as control. Reference standards are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzylalcohol
3. Trinitrobenzoic Acid
4. 4-Amino-2,6-Dinitrotoluene
5. 2-Amino-4,6-Dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2,6'-Tetranitro-4,4'-azoxytoluene

Figure 19 follows

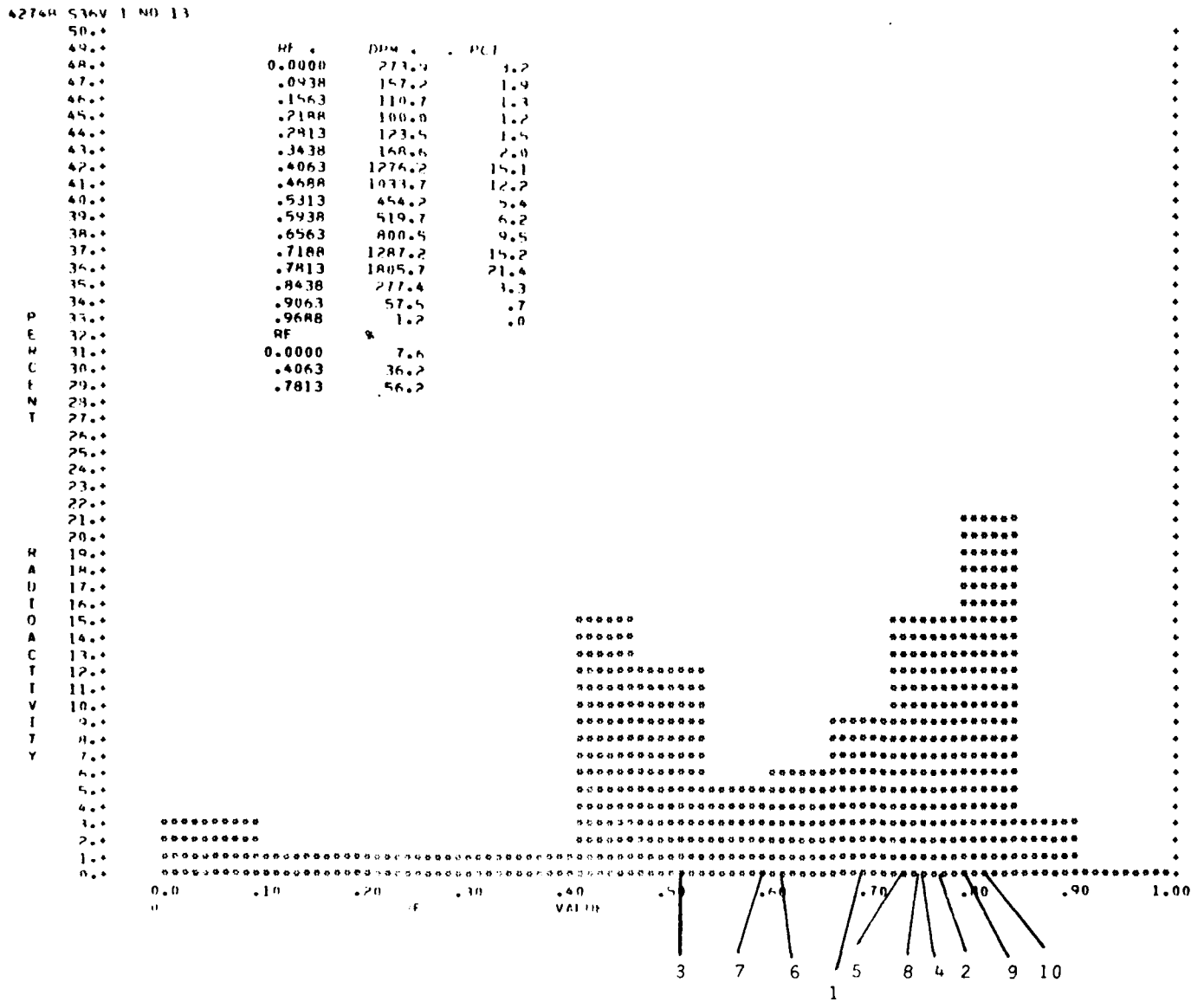

Figure 19-a-I: Oral Treatment, Incubation with Water, Solvent I

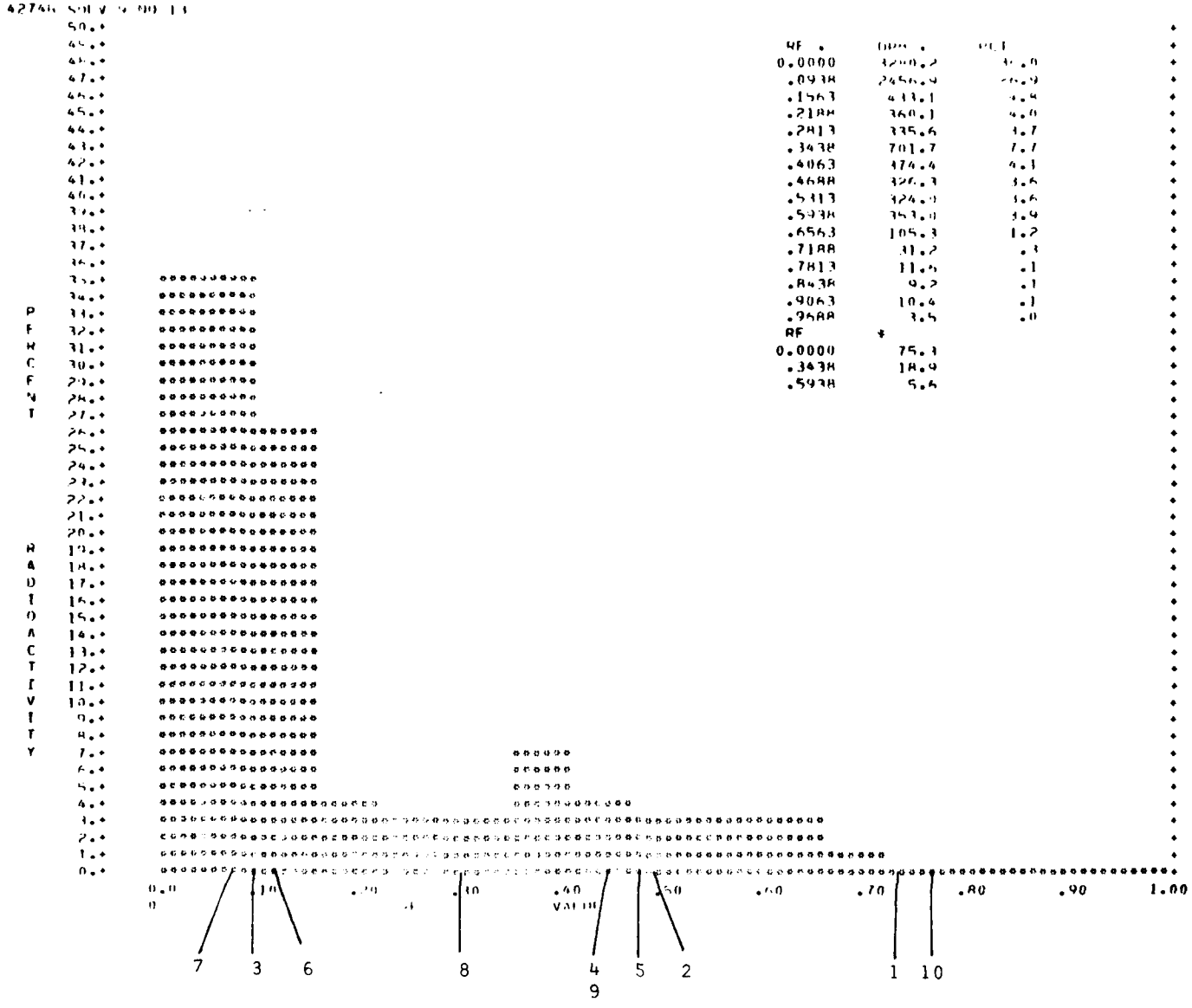

Figure 19-a-IX: Oral Treatment, Incubation with Water, Solvent IX

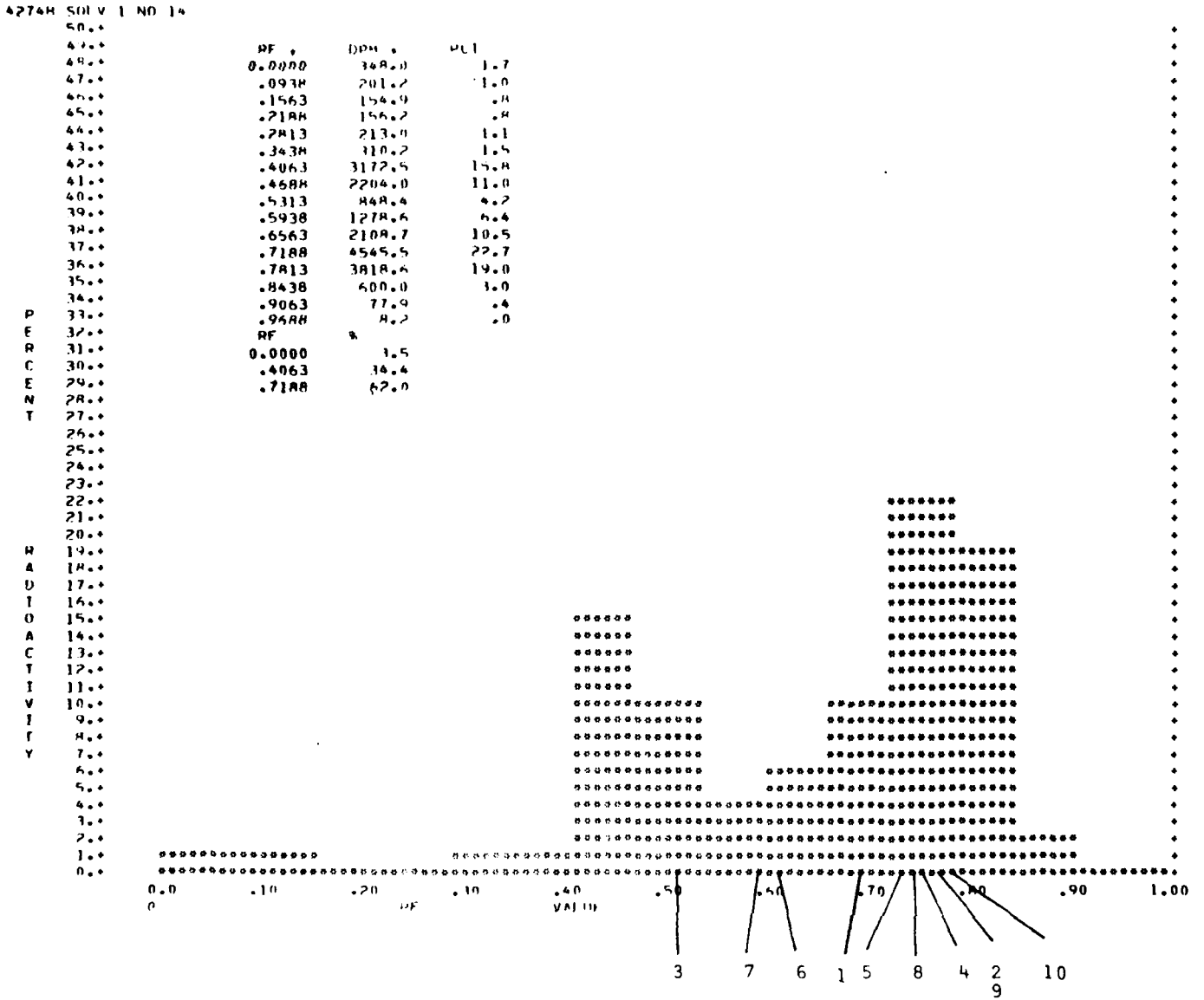

Figure 19-b-I: Oral Treatment, Incubation with β-Glucuronidase, Solvent I

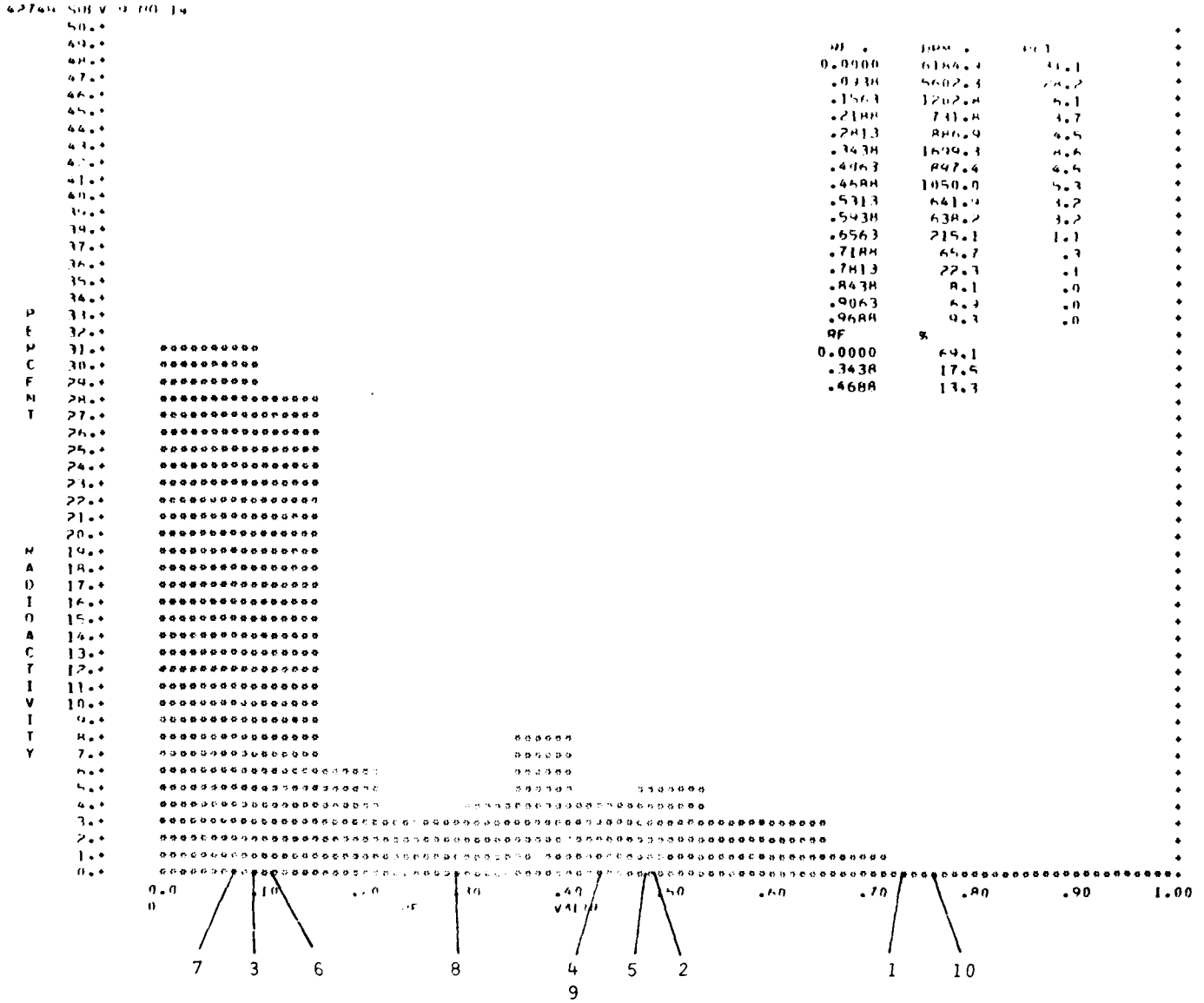

Figure 19-b-IX: Oral Treatment, Incubation with β-Glucuronidase, Solvent IX

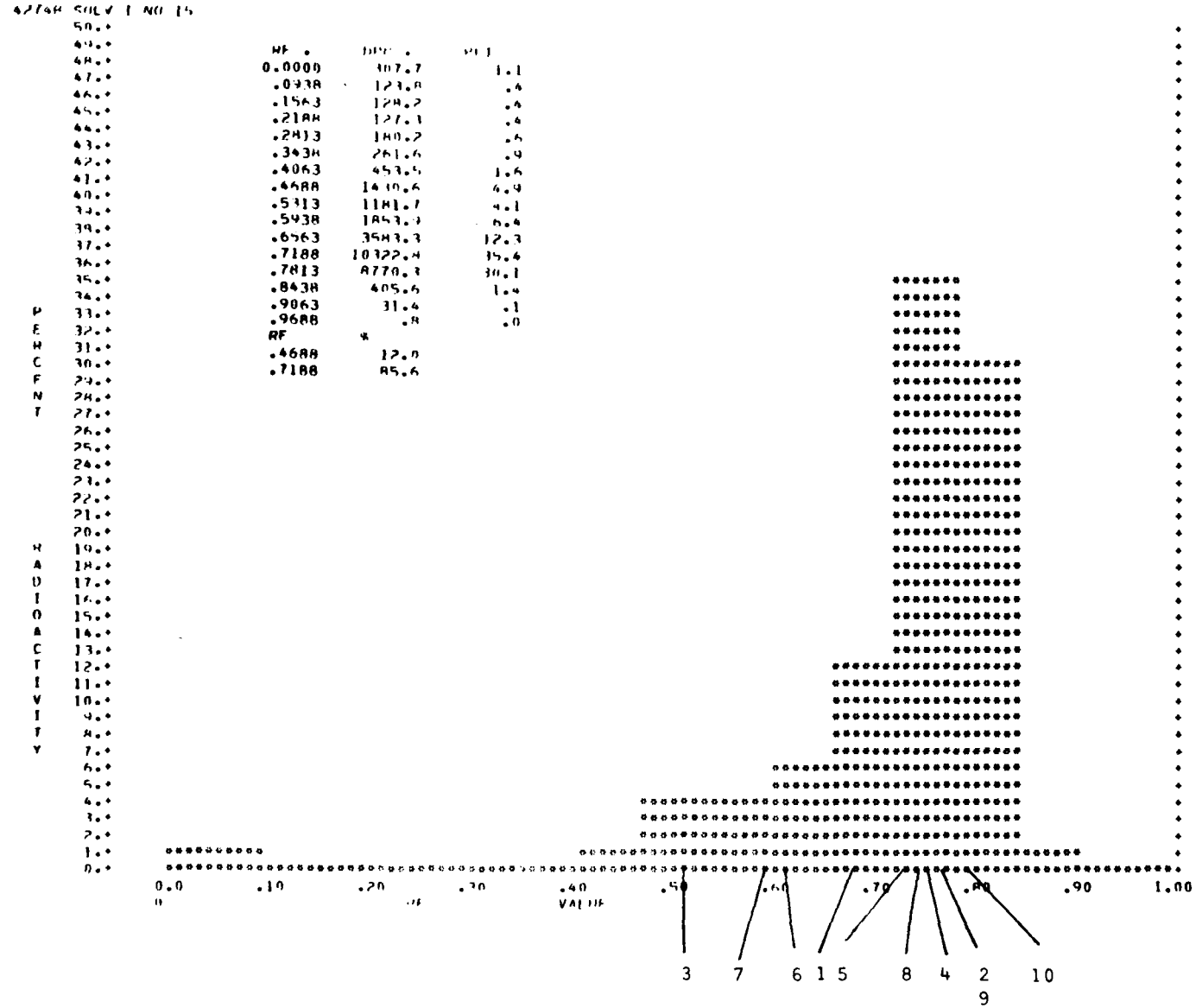

Figure 19-c-I: Intratracheal Instillation, Incubation with Water, Solvent I

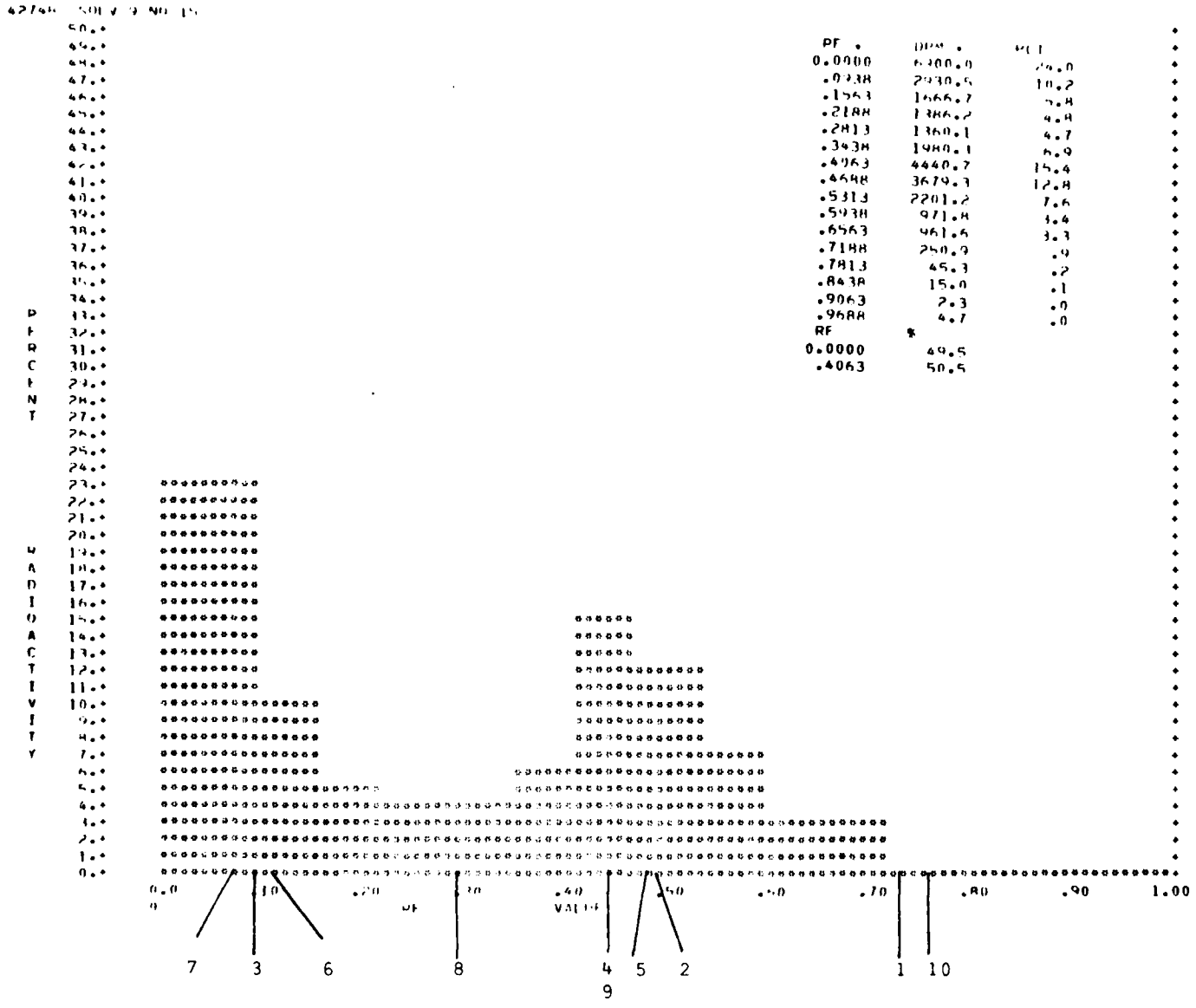

Figure 19-c-IX: Intratracheal Instillation, Incubation with Water, Solvent IX

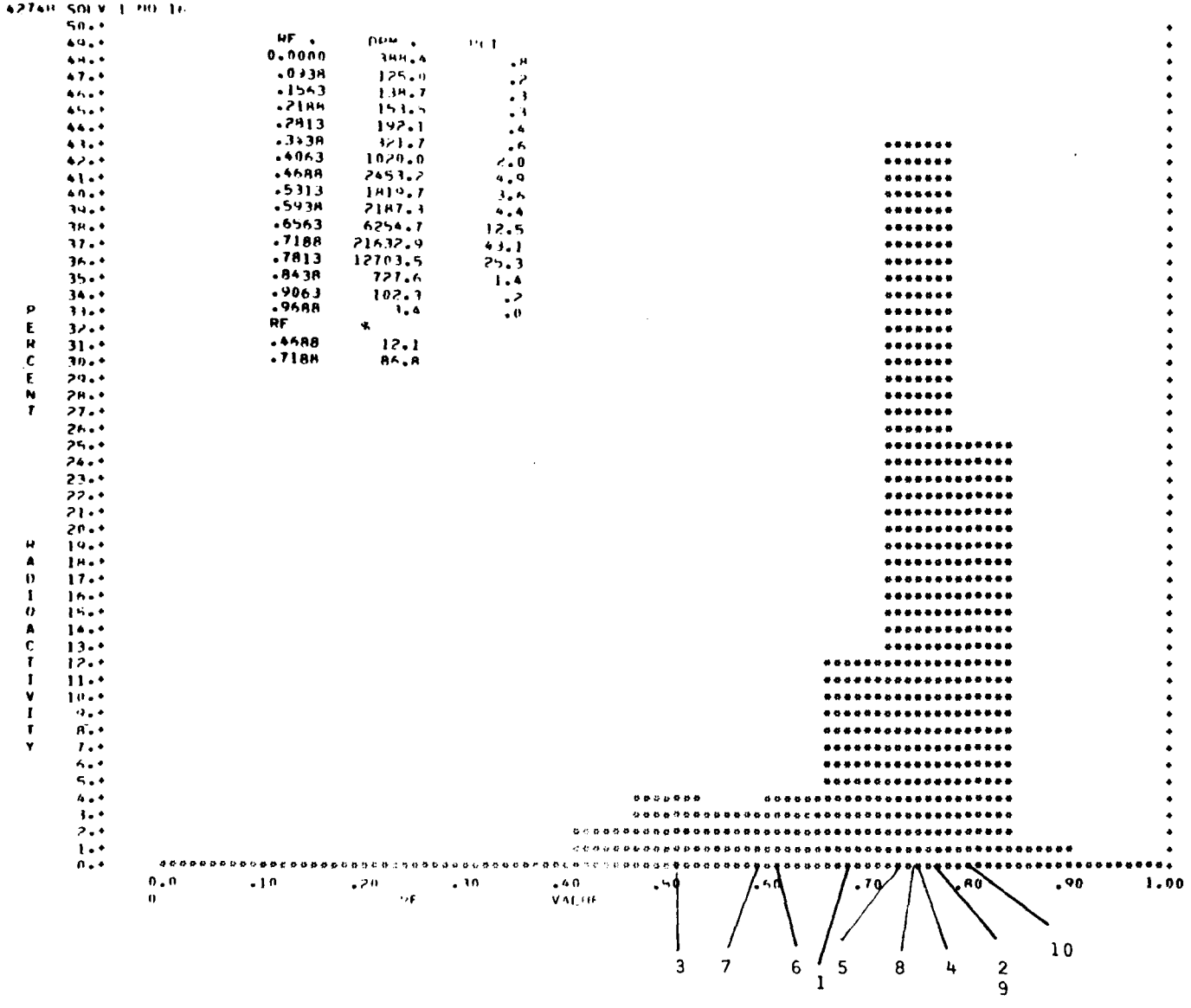

Figure 19-d-I: Intratracheal Instillation, Incubation with β-Glucuronidase, Solvent I

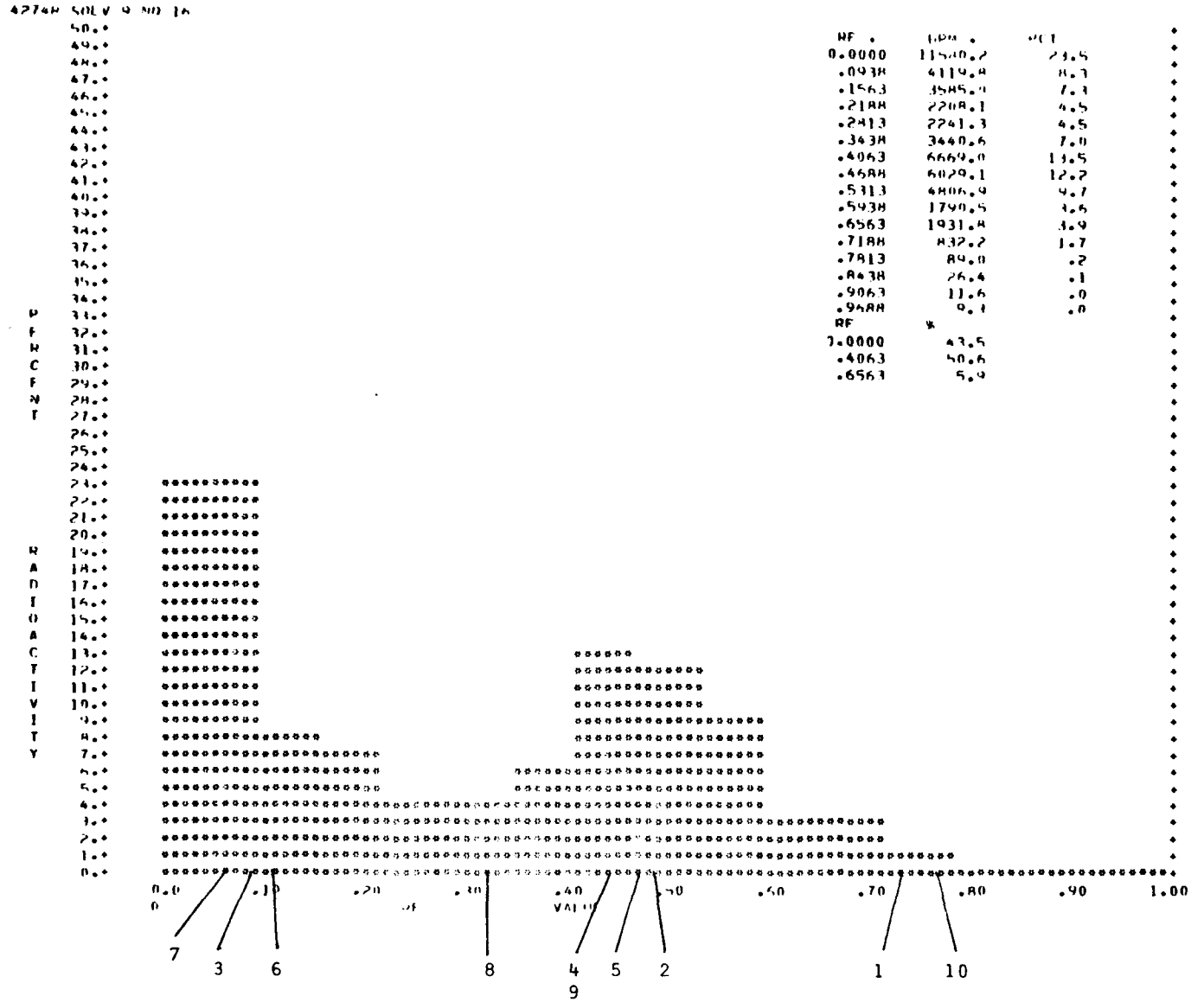

Figure 19-d-IX: Intratracheal Instillation, Incubation with β-Glucuronidase, Solvent IX

Figure 20: TLC of Ethyl Acetate-Extractable Products Obtained from 24-Hr Urine of Male Mice Treated Orally or Dermally with ^{14}C-TNT. Samples of urine were incubated with acetate buffer and β-glucuronidase before extraction with ethyl acetate. Incubation with acetate buffer and water served as control. Reference standards are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzylalcohol
3. Trinitrobenzoic Acid
4. 4-Amino-2,6-Dinitrotoluene
5. 2-Amino-4,6-Dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2,6'-Tetranitro-4,4'-azoxytoluene

Figure 20 follows

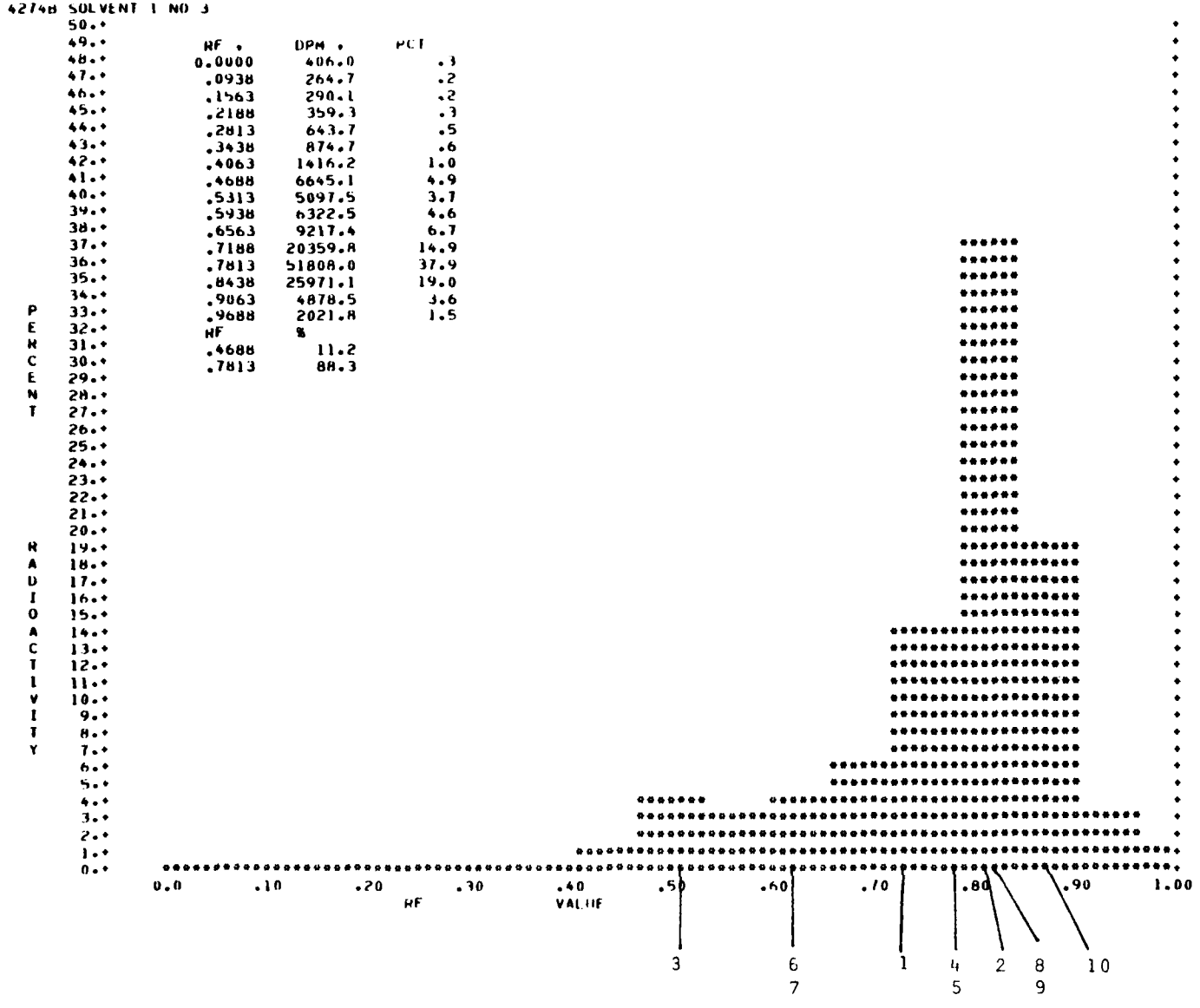

Figure 20-a-I: Oral Treatment, Incubation with Water, Solvent I

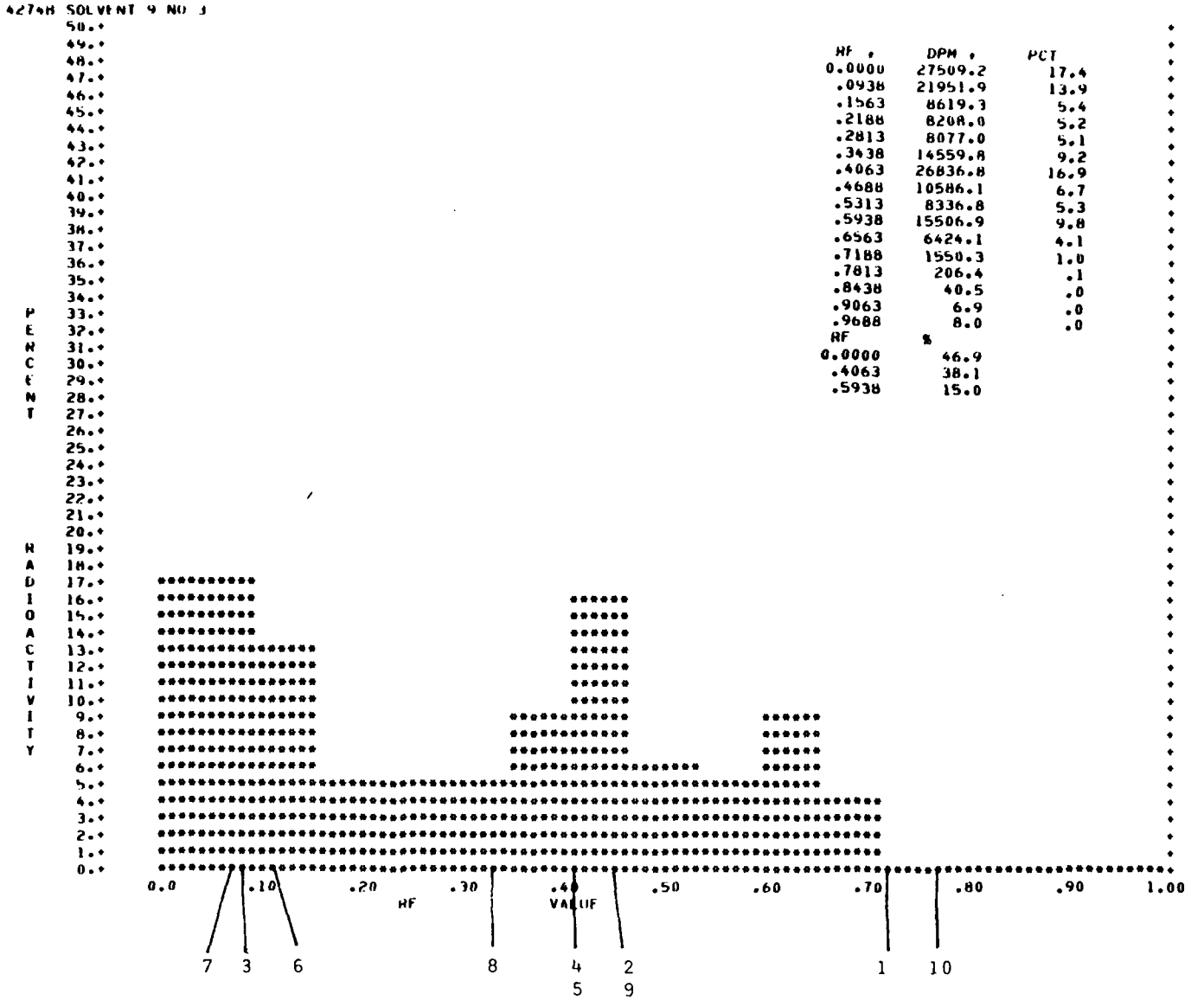

Figure 20-a-IX: Oral Treatment, Incubation with Water, Solvent IX

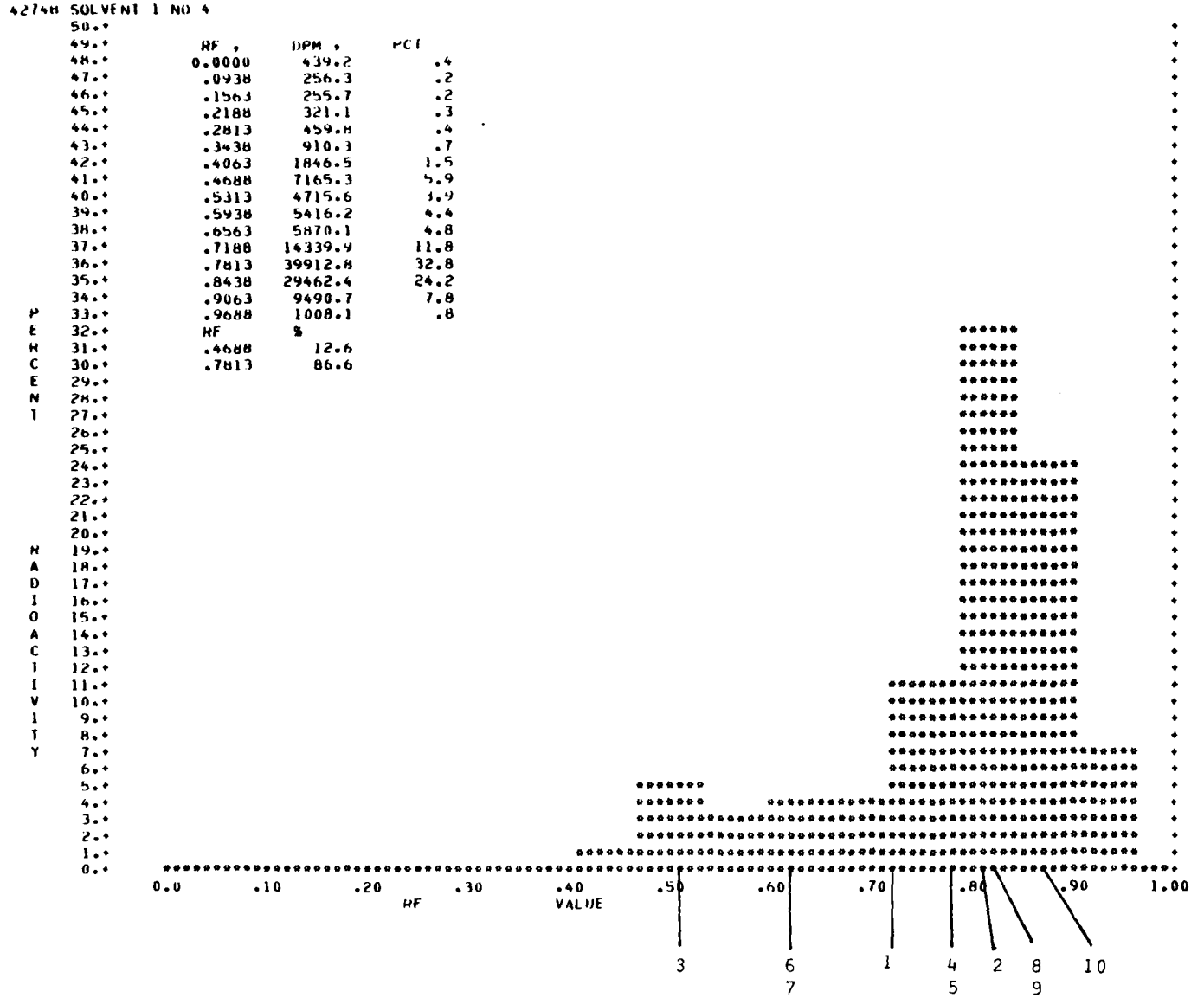

Figure 20-b-1: Oral Treatment, Incubation with β-Glucuronidase, Solvent I

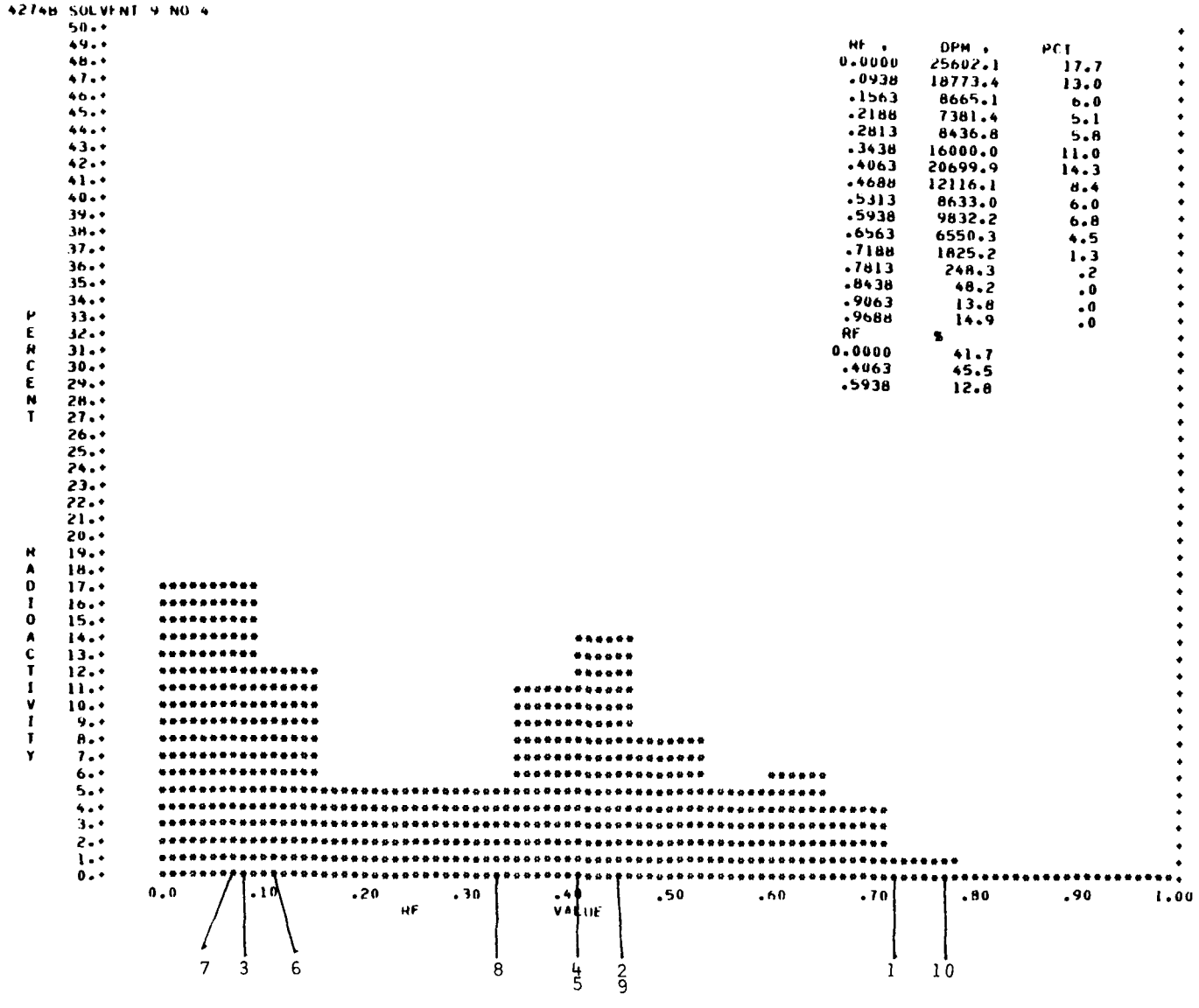

Figure 20-b-IX: Oral Treatment, Incubation with β-Glucuronidase, Solvent IX

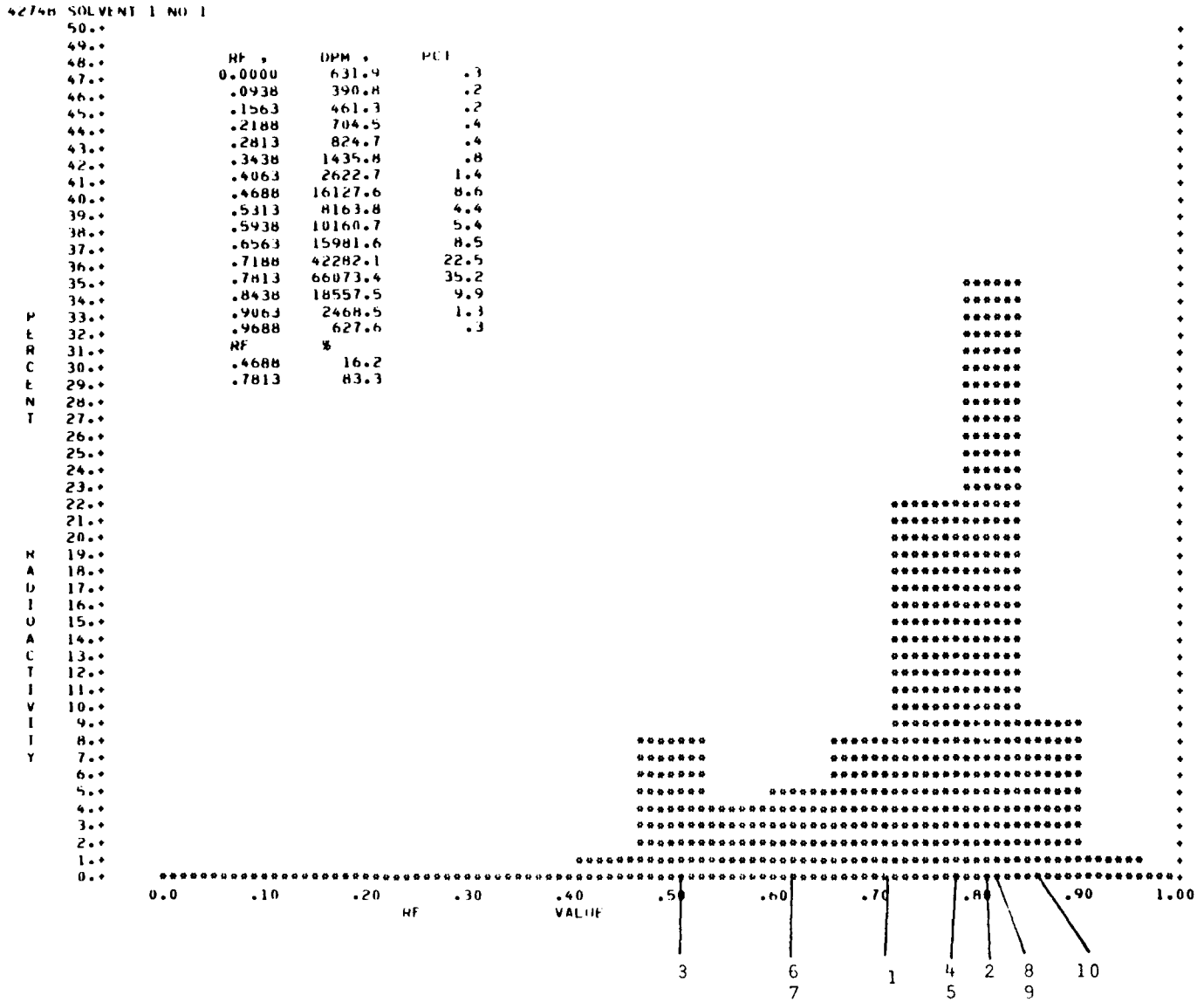

Figure 20-c-I: Dermal Application, Incubation with Water, Solvent I

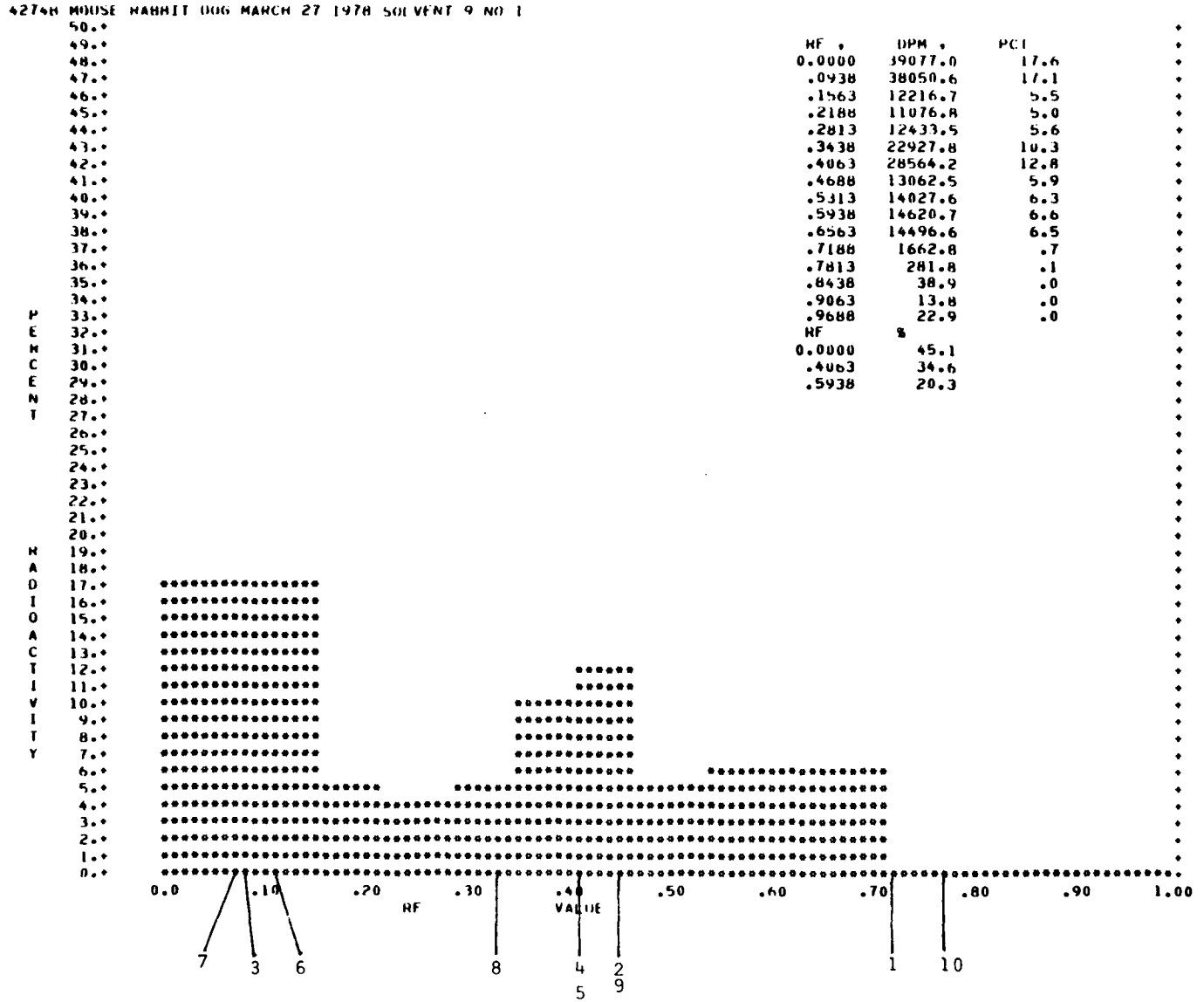

Figure 20-c-IX: Dermal Application, Incubation with Water, Solvent IX

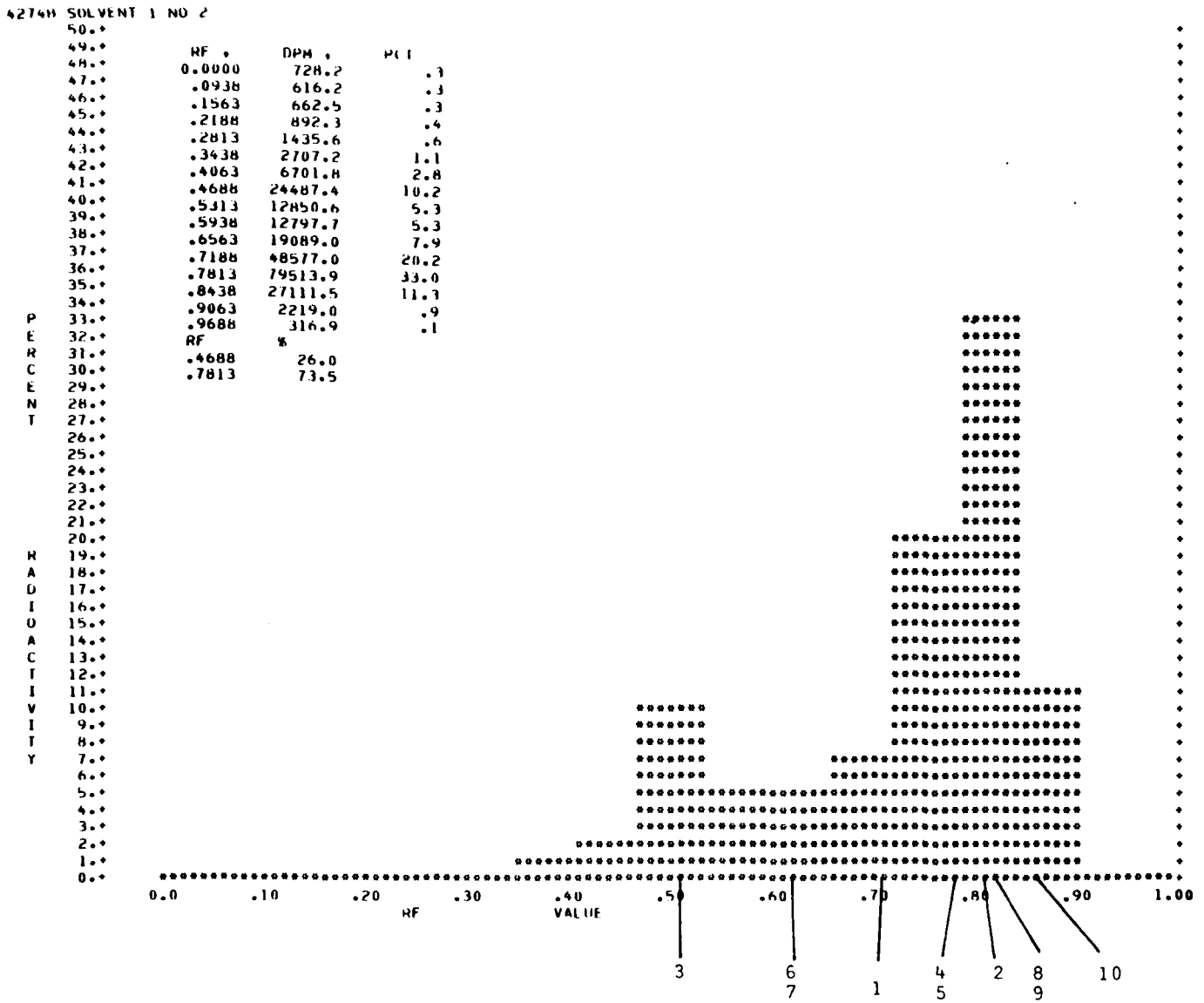

Figure 20-d-I: Dermal Application, Incubation with β-Glucuronidase, Solvent I

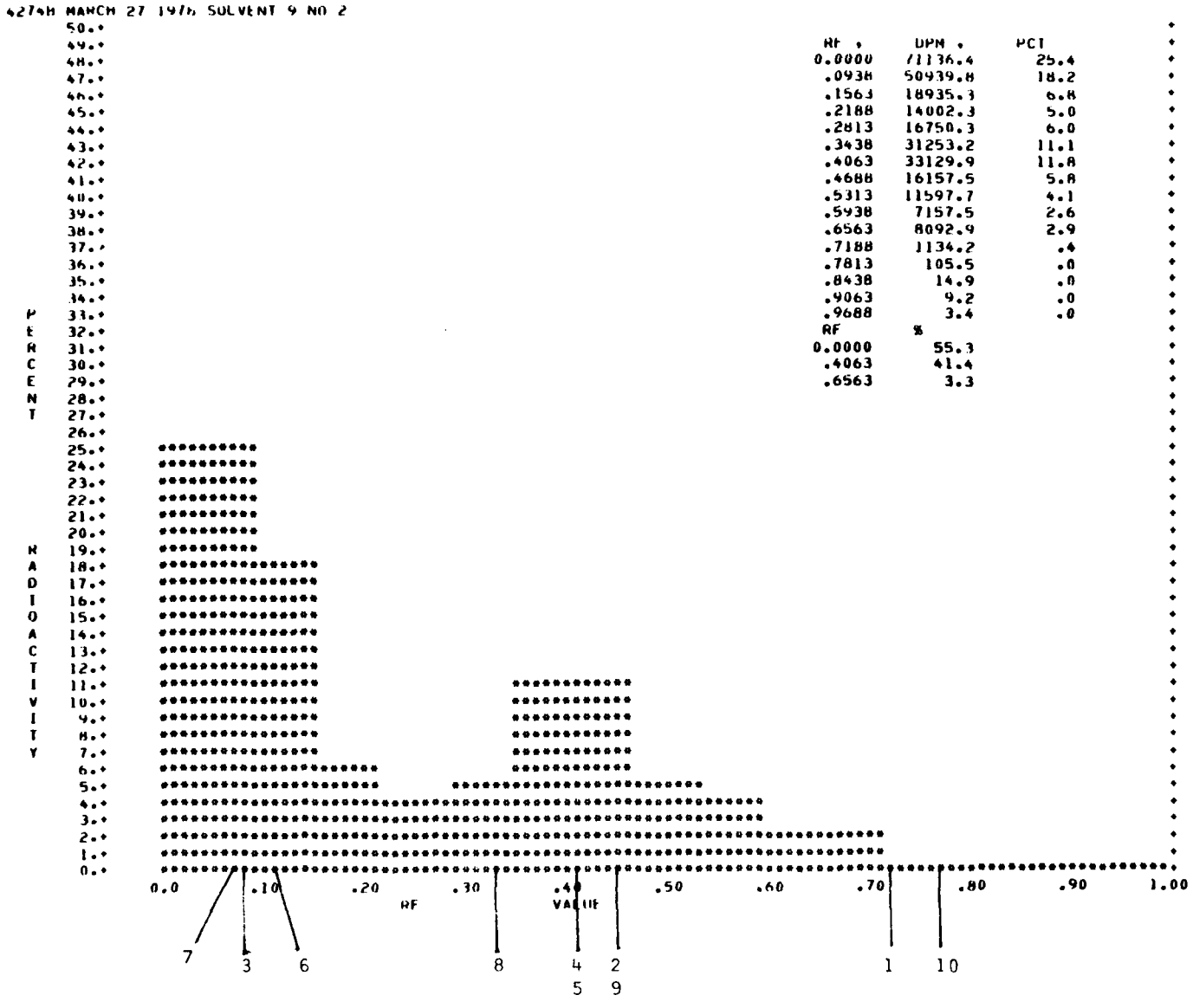

Figure 20-d-IX: Dermal Application, Incubation with β-Glucuronidase, Solvent IX

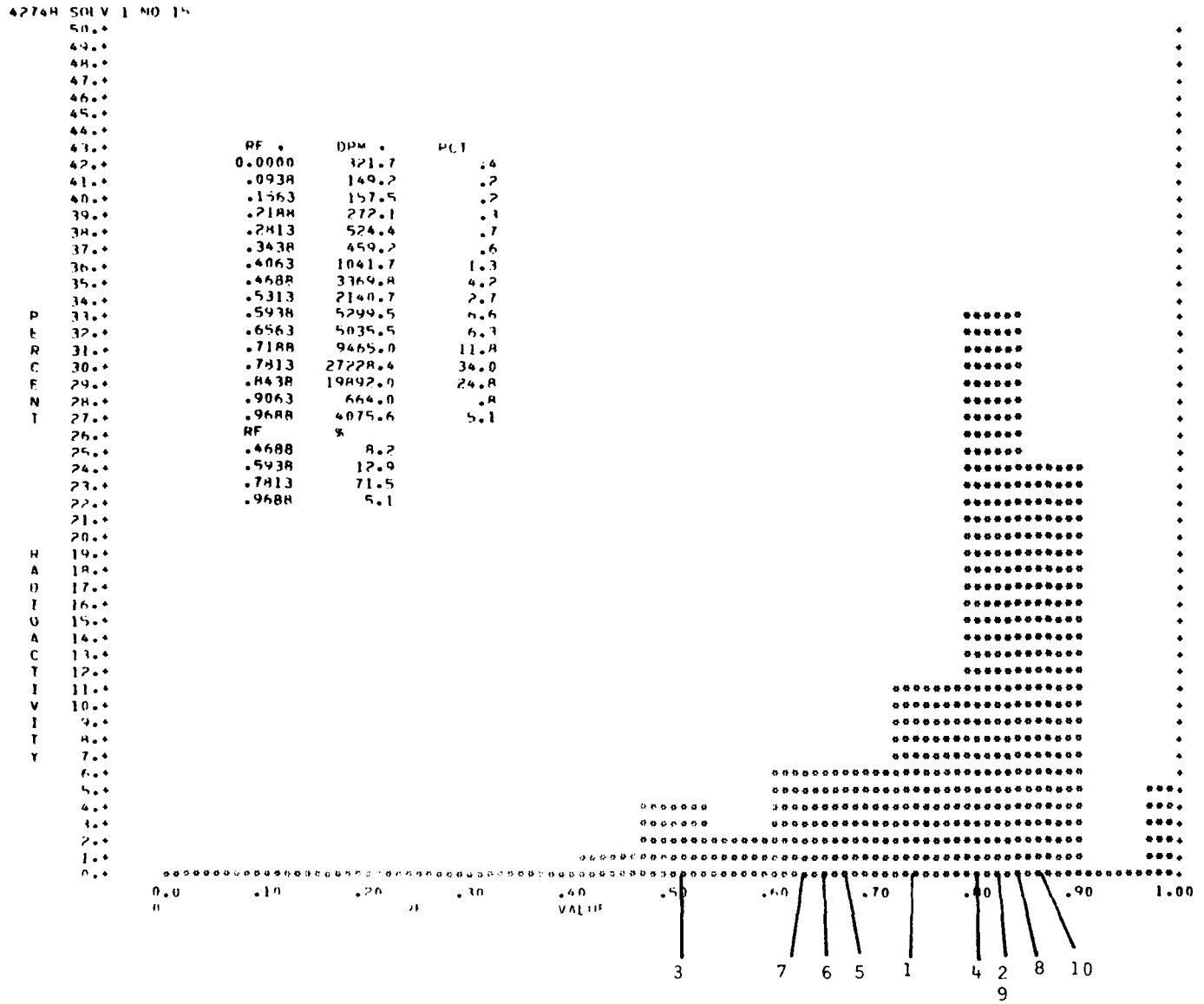

Figure 20-e-I: Oral Treatment, Incubation with Water, Solvent I

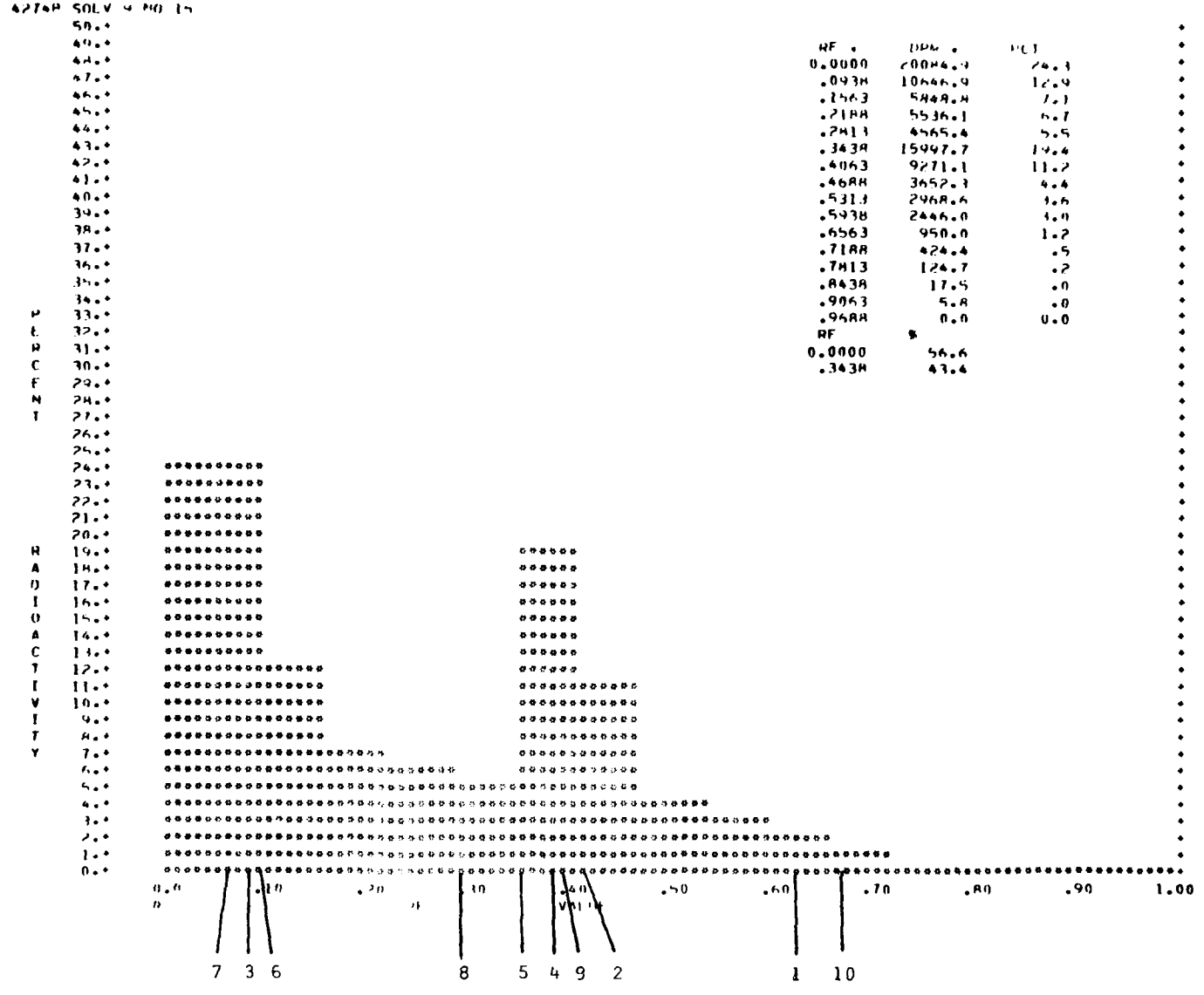

Figure 20-e-IX: Oral Treatment, Incubation with Water, Solvent IX

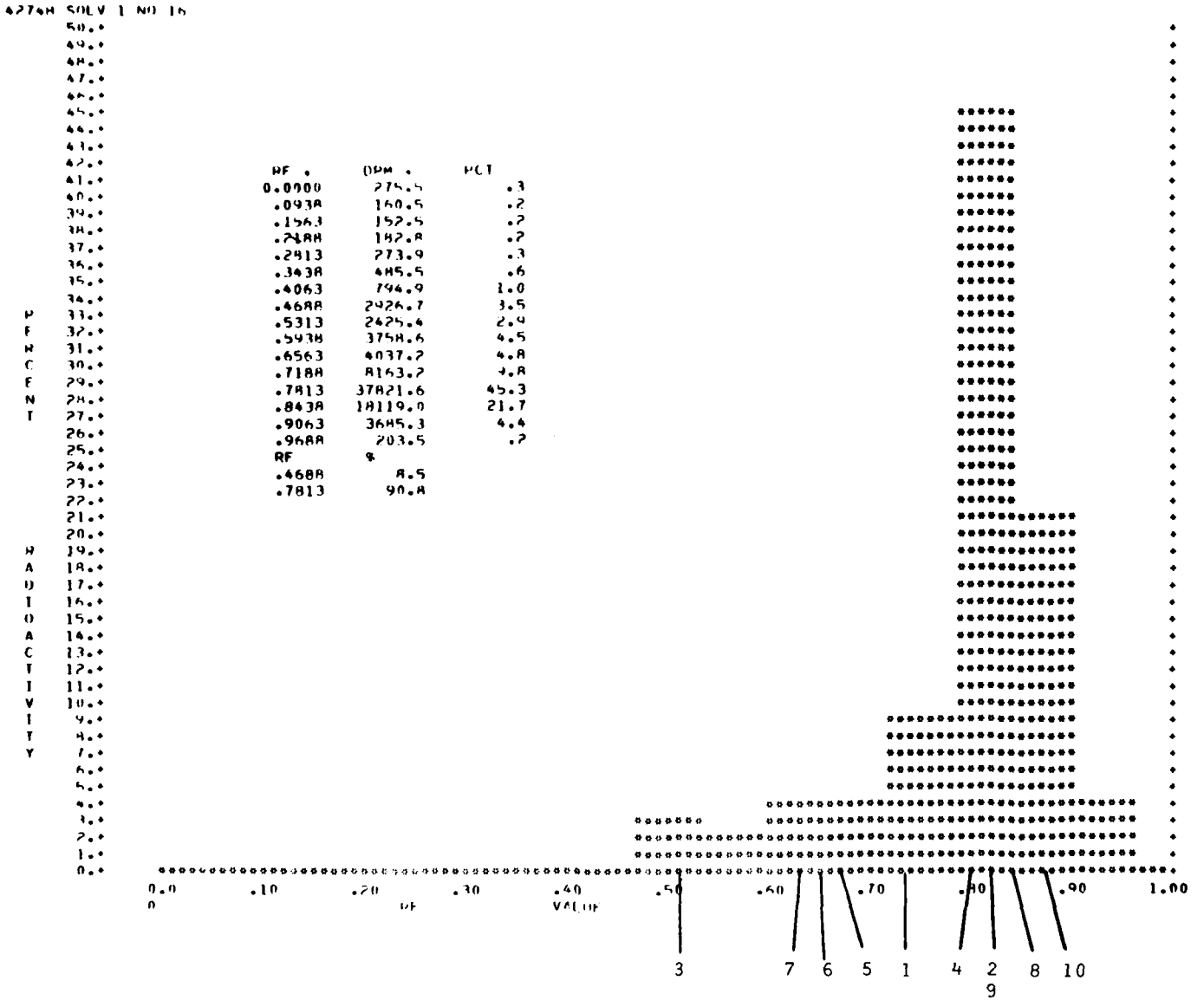

Figure 20-f-I: Oral Treatment, Incubation with β-Glucuronidase, Solvent I

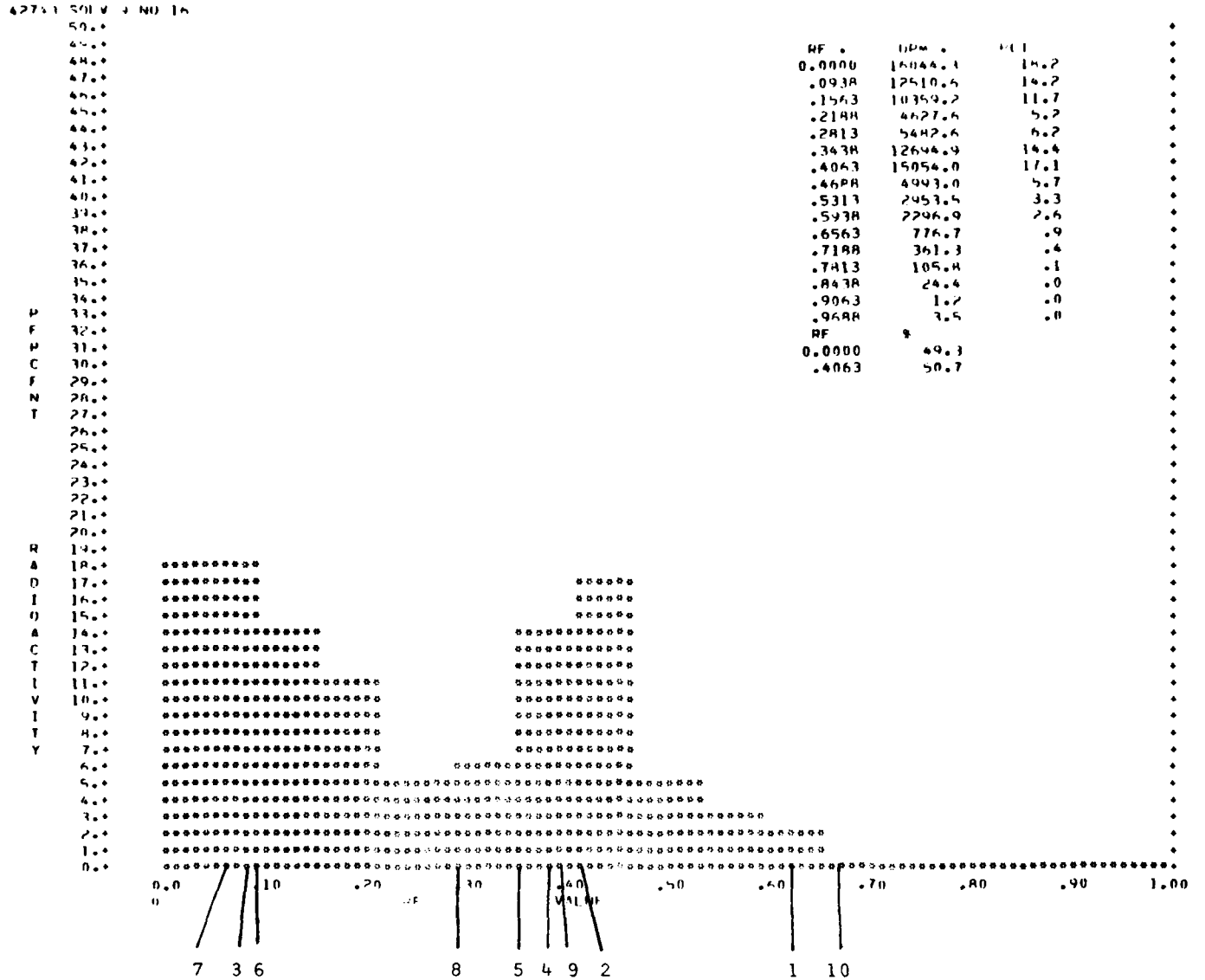

Figure 20-f-IX: Oral Treatment, Incubation with β-Glucuronidase, Solvent IX

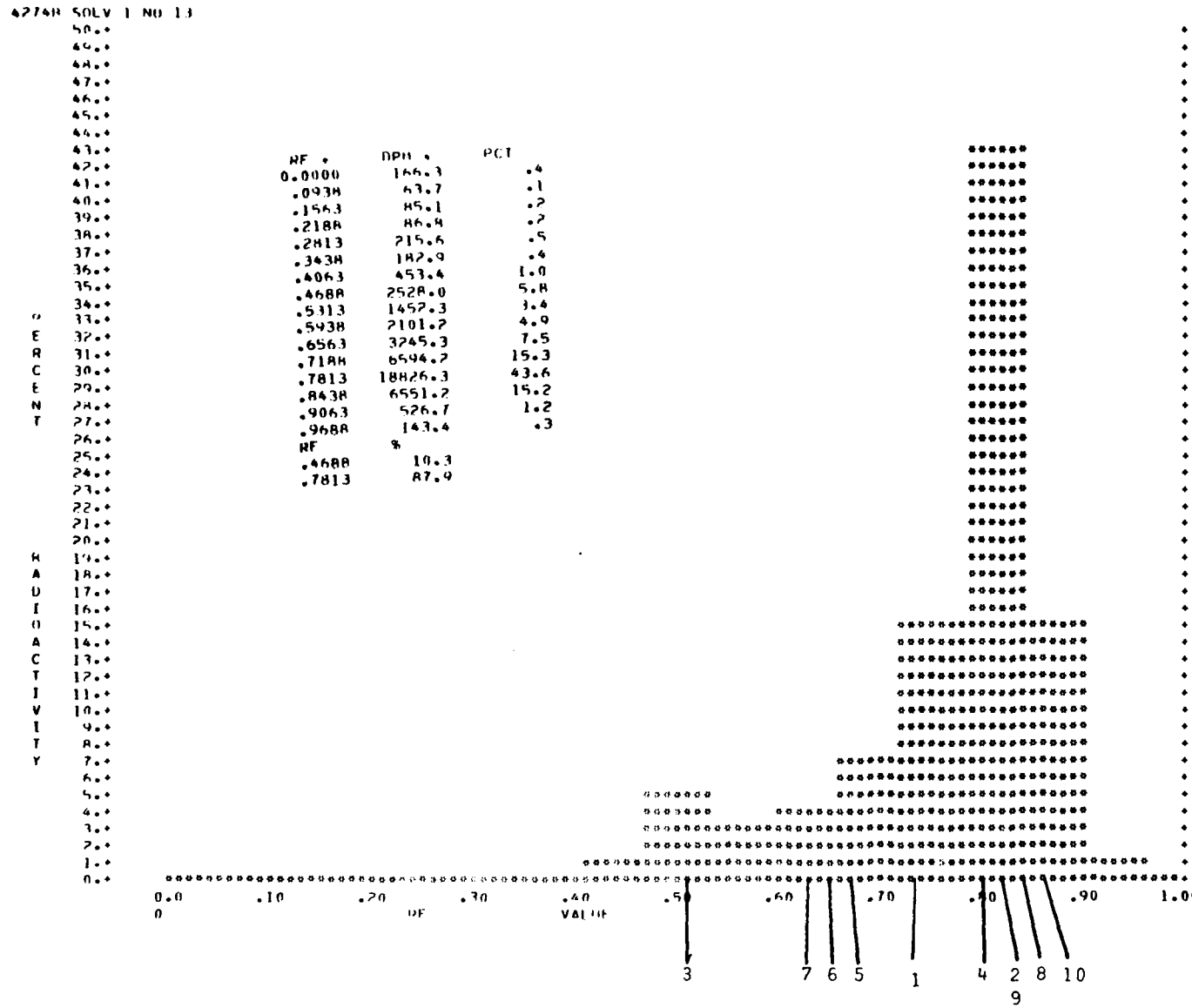

Figure 20-g-I: Dermal Application, Incubation with Water, Solvent I

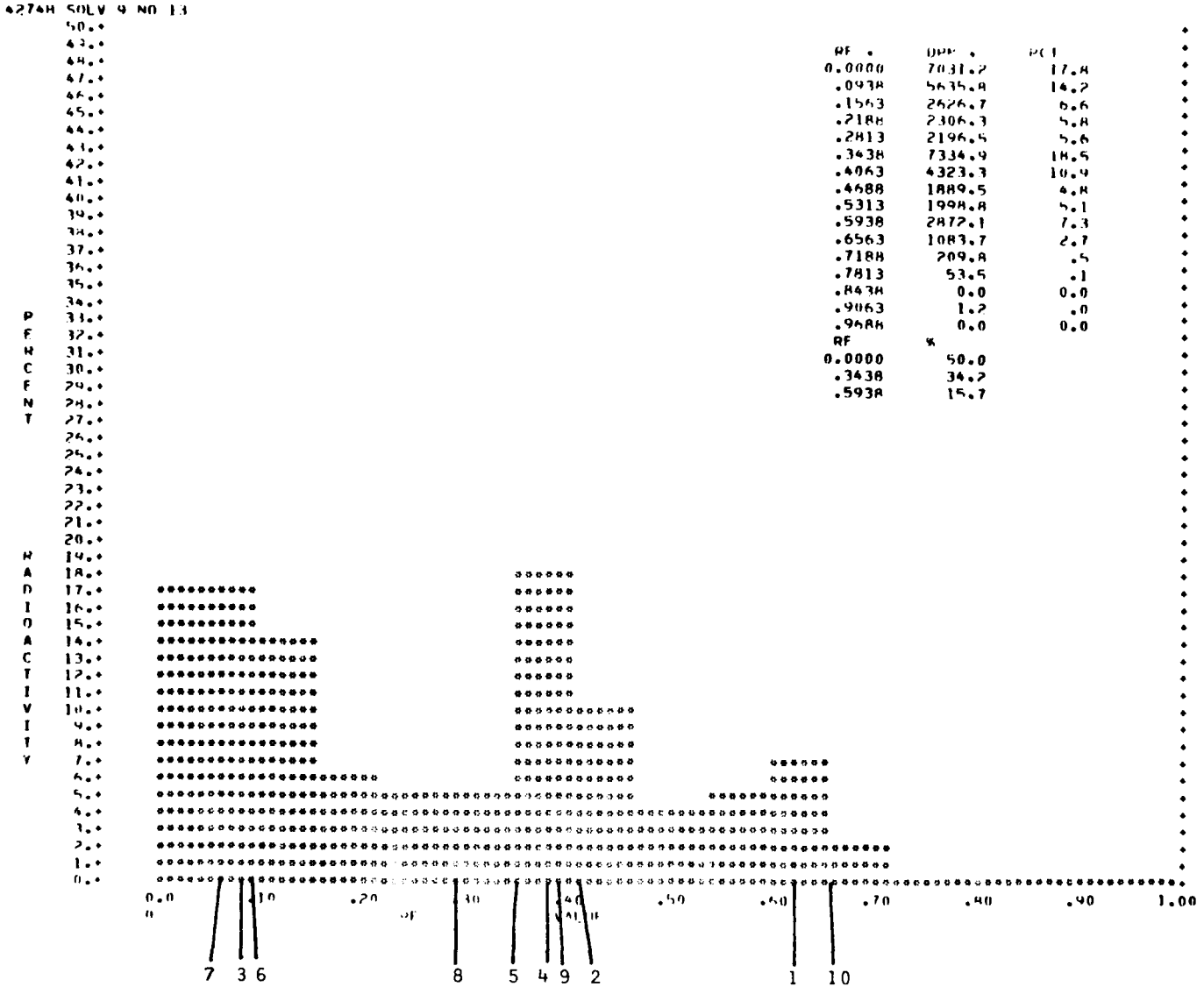

Figure 20-g-IX: Dermal Application, Incubation with Water, Solvent IX

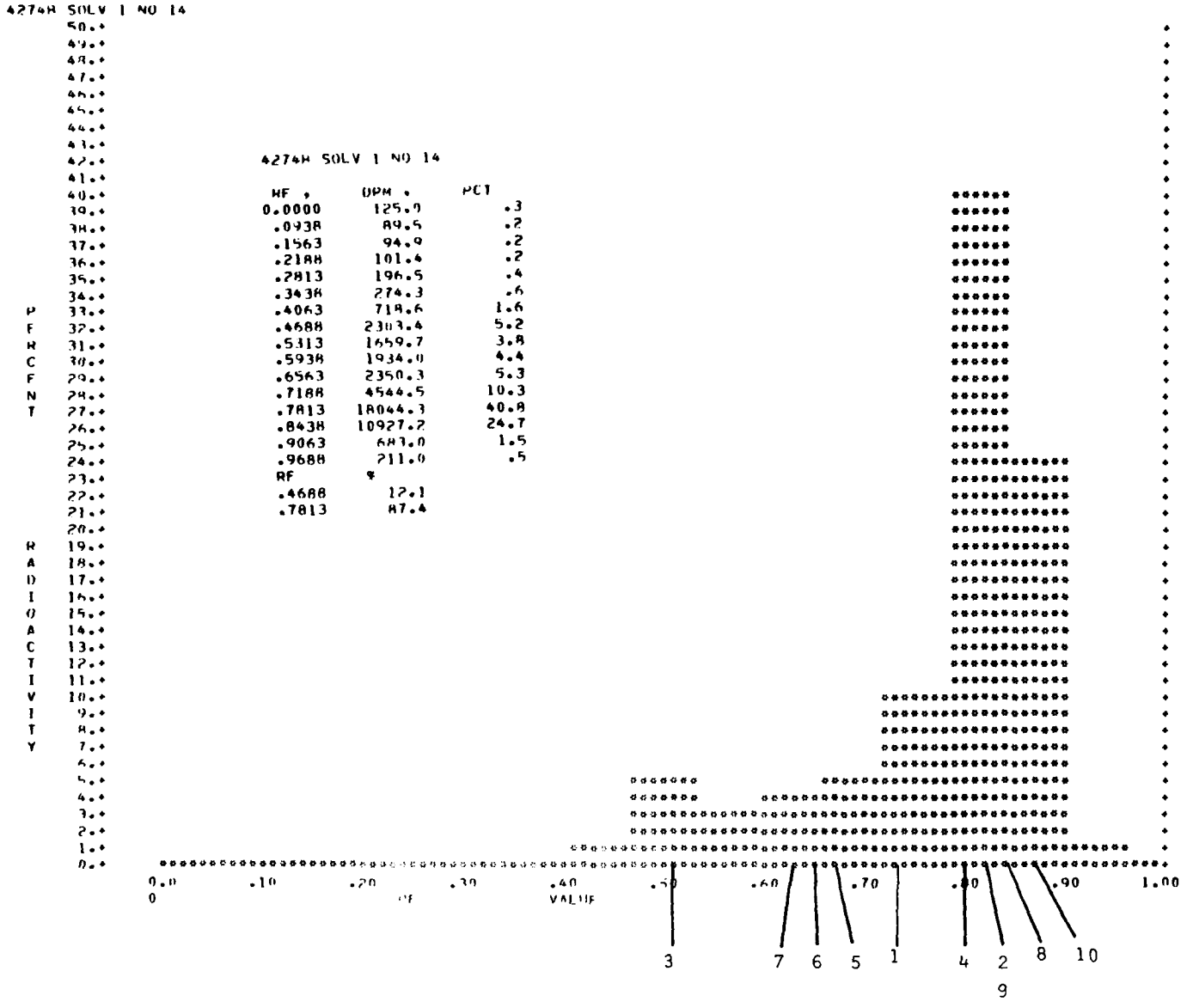

Figure 20-h-I: Dermal Application, Incubation with β-Glucuronidase, Solvent I

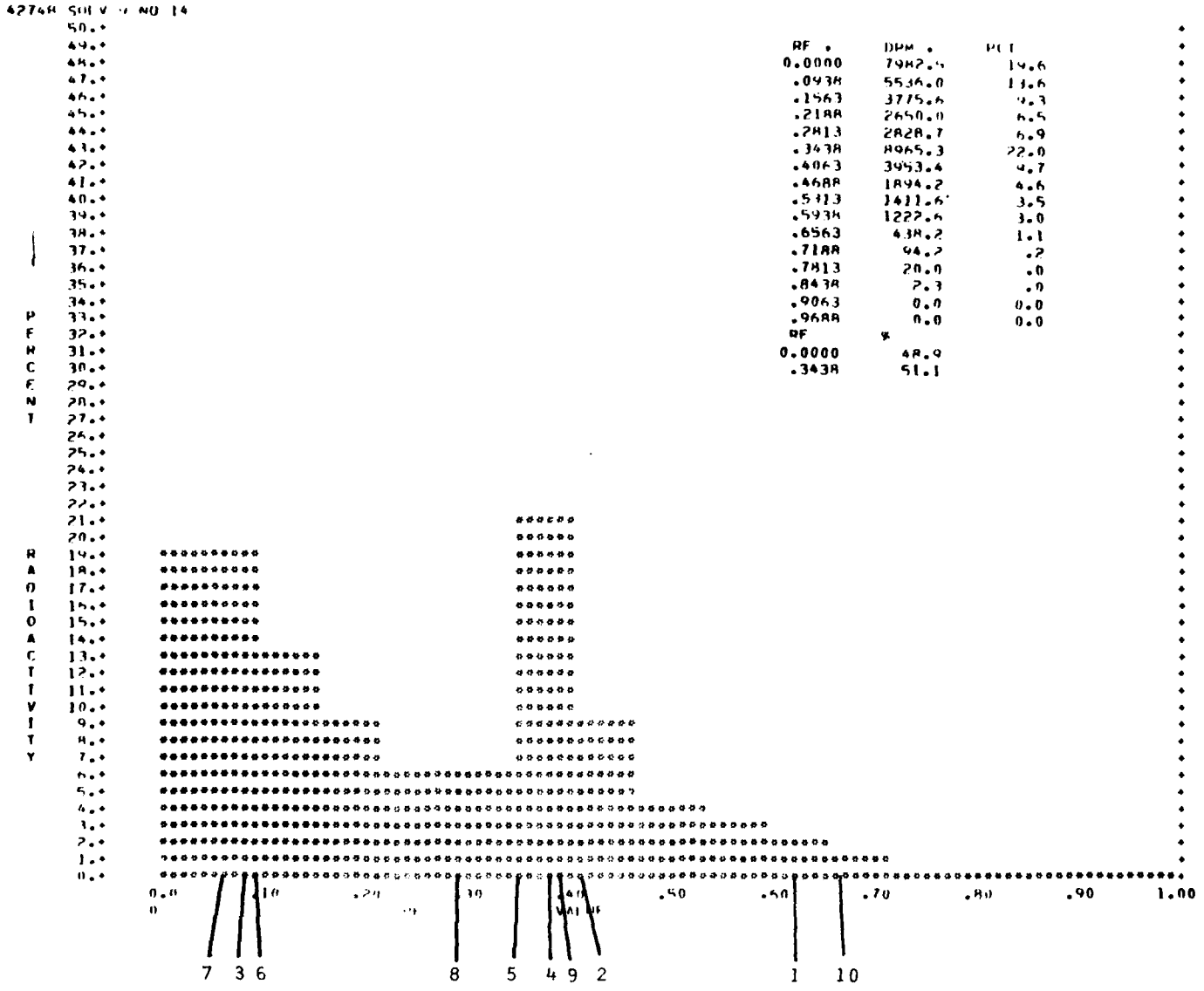

Figure 20-h-IX: *Dermal Application, Incubation with β-Glucuronidase, Solvent IX*

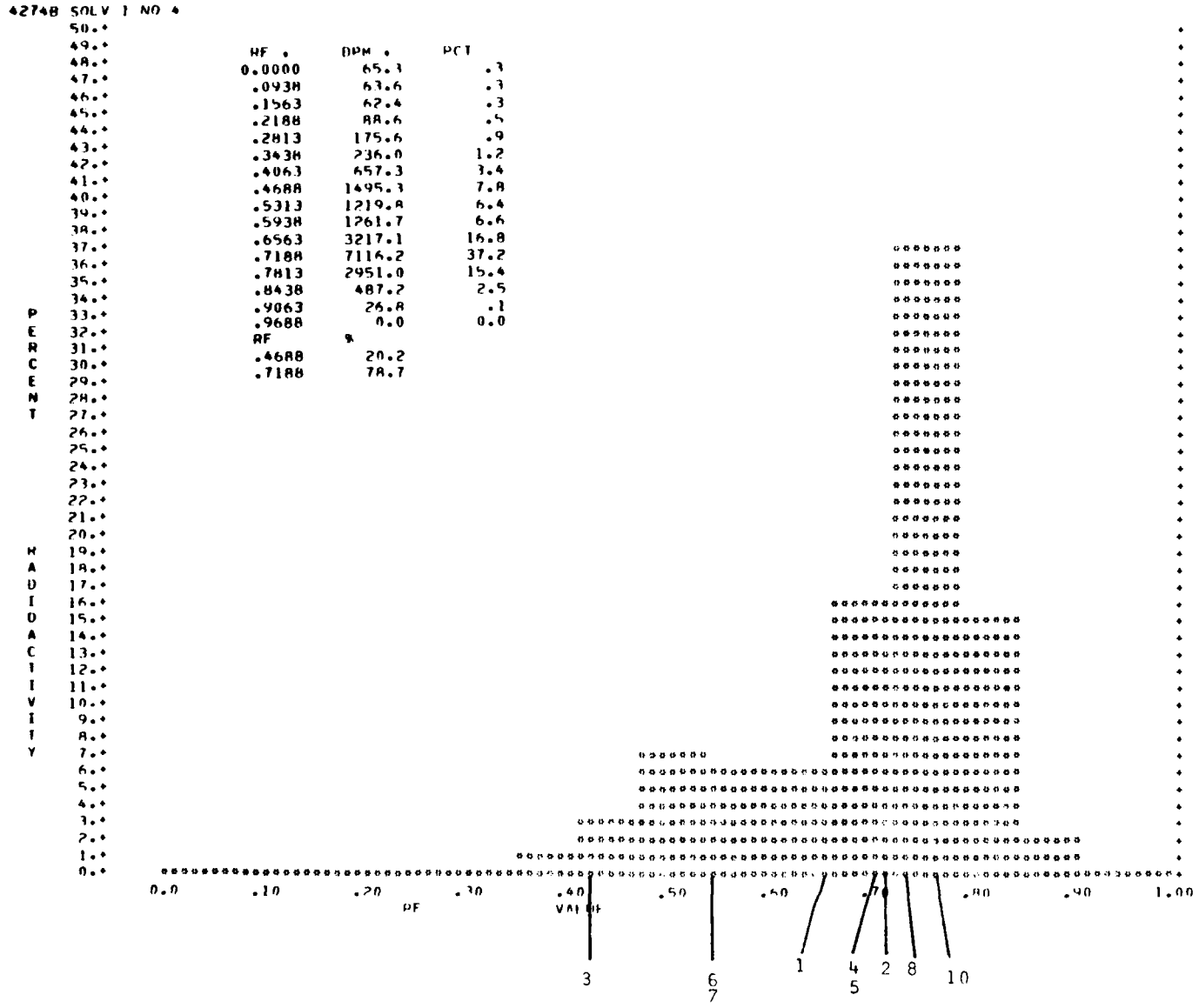

Figure 20-k-I: Oral Treatment, Incubation with β-Glucuronidase, Solvent I

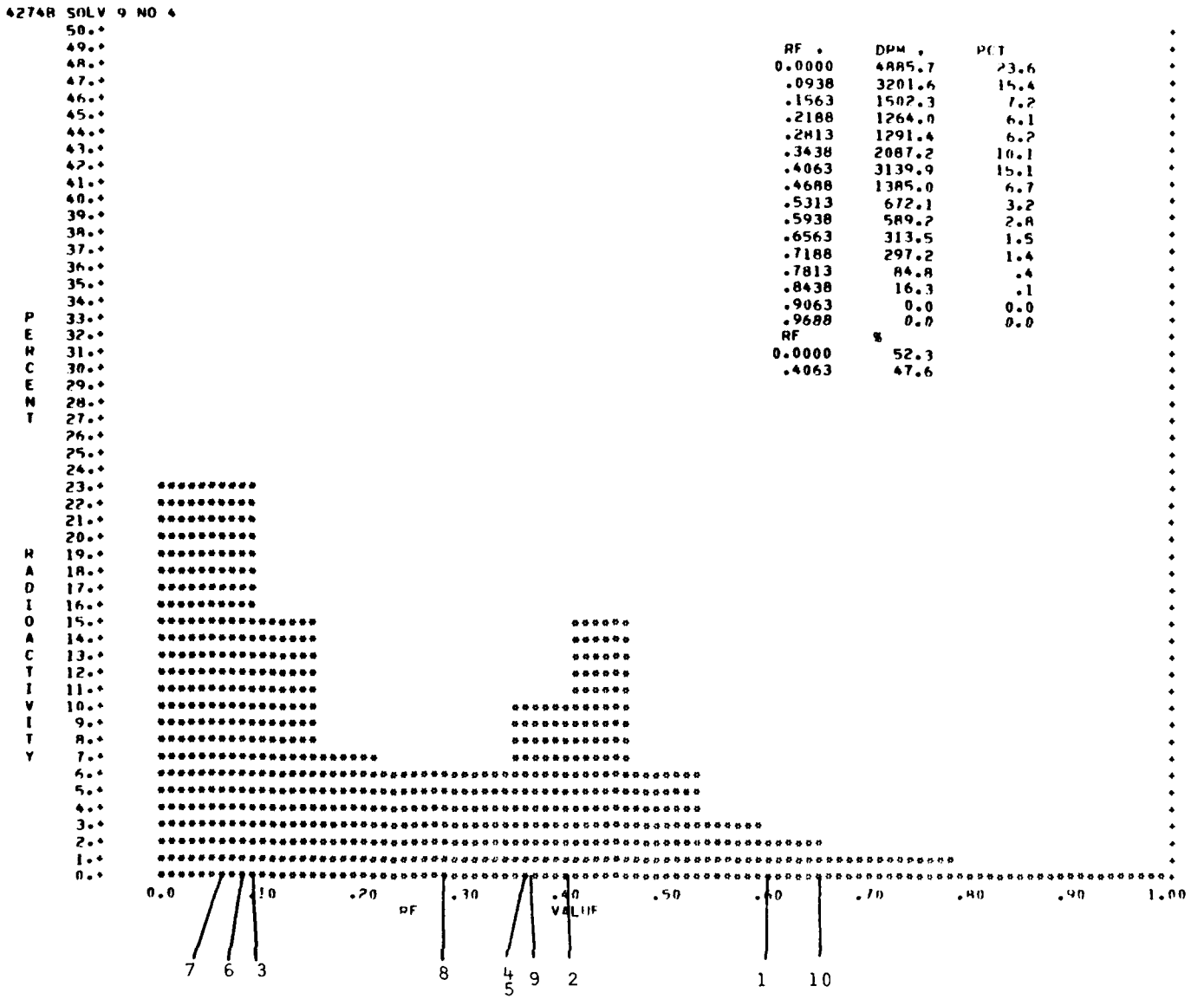

Figure 20-k-IX: Oral Treatment, Incubation with β-Glucuronidase, Solvent IX

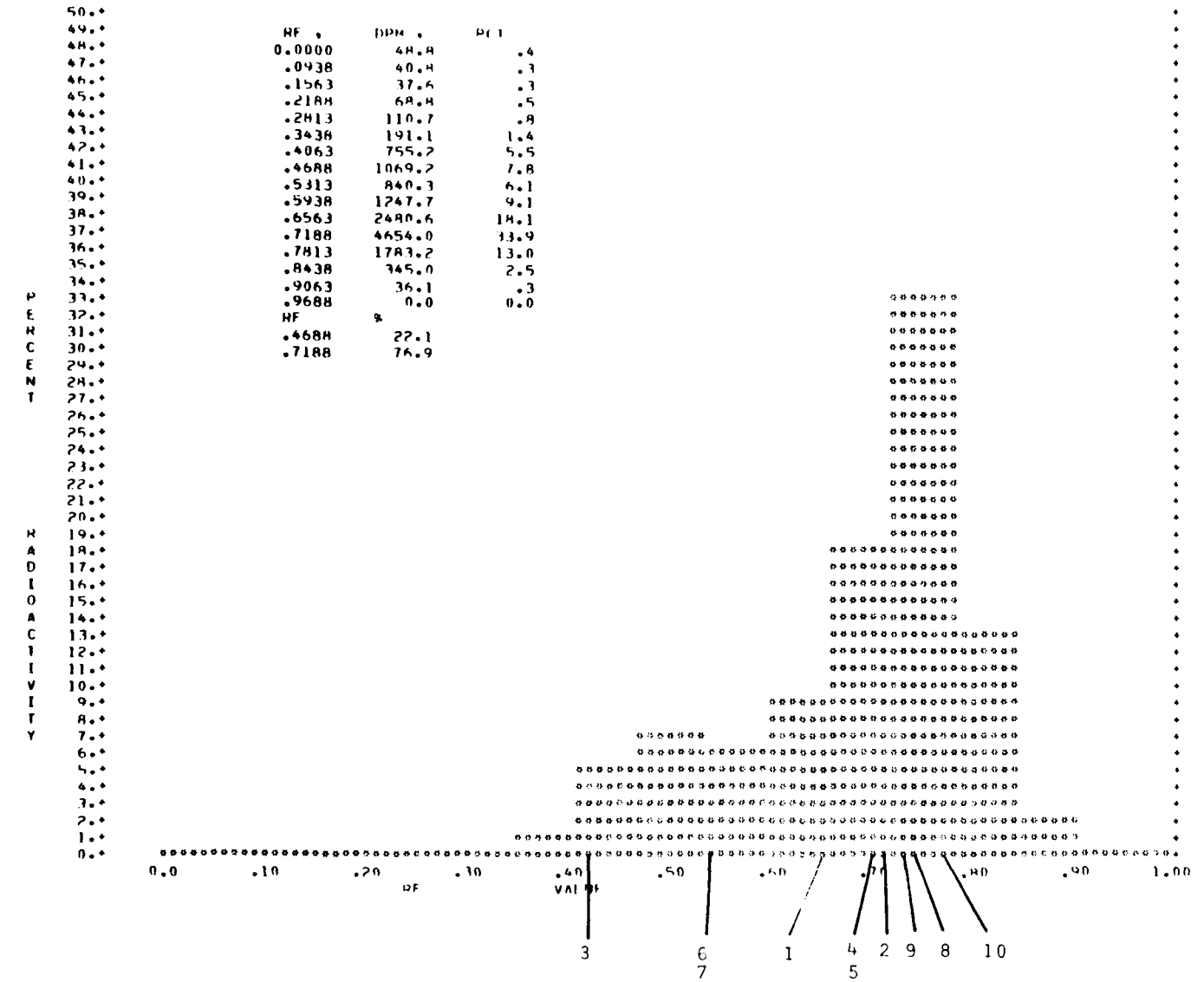

Figure 20-1-I: Dermal Application, Incubation with ß-Glucuronidase, Solvent I

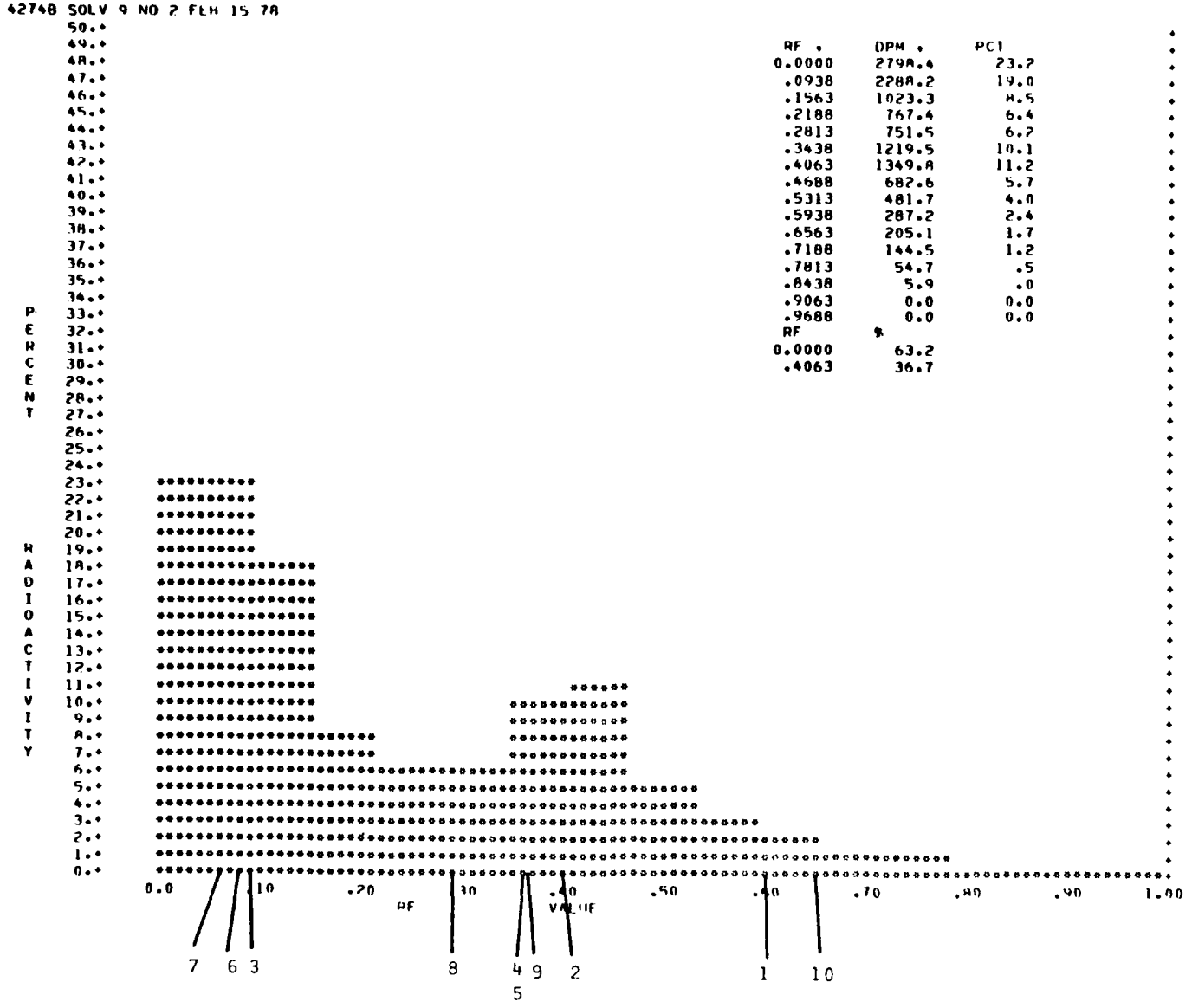

Figure 20-1-IX: Dermal Application, Incubation with β-Glucuronidase, Solvent IX

Figure 21: TLC of Ethyl Acetate-Extractable Products Obtained from 24-Hr Urine of Male Rabbits Treated Orally or Dermally with ^{14}C-TNT. Samples of urine were incubated with acetate buffer and β-glucuronidase before extraction with ethyl acetate. Incubation with acetate buffer and water served as control. Reference standards are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzylalcohol
3. Trinitrobenzoic Acid
4. 4-Amino-2,6-Dinitrotoluene
5. 2-Amino-4,6-Dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2,6'-Tetranitro-4,4'-azoxytoluene

Figure 21 follows

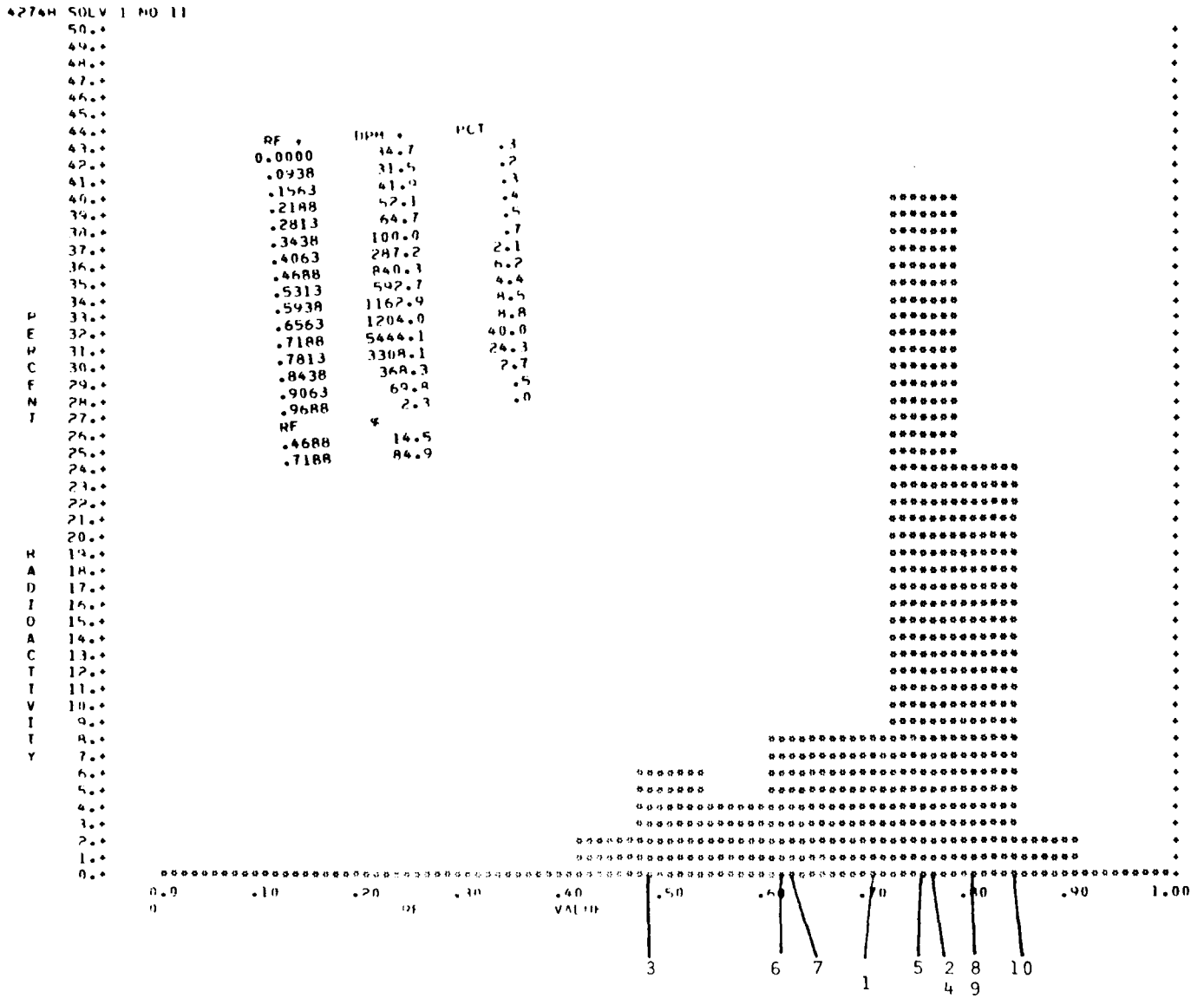

Figure 21-a-I: Oral Treatment, Incubation with Water, Solvent I

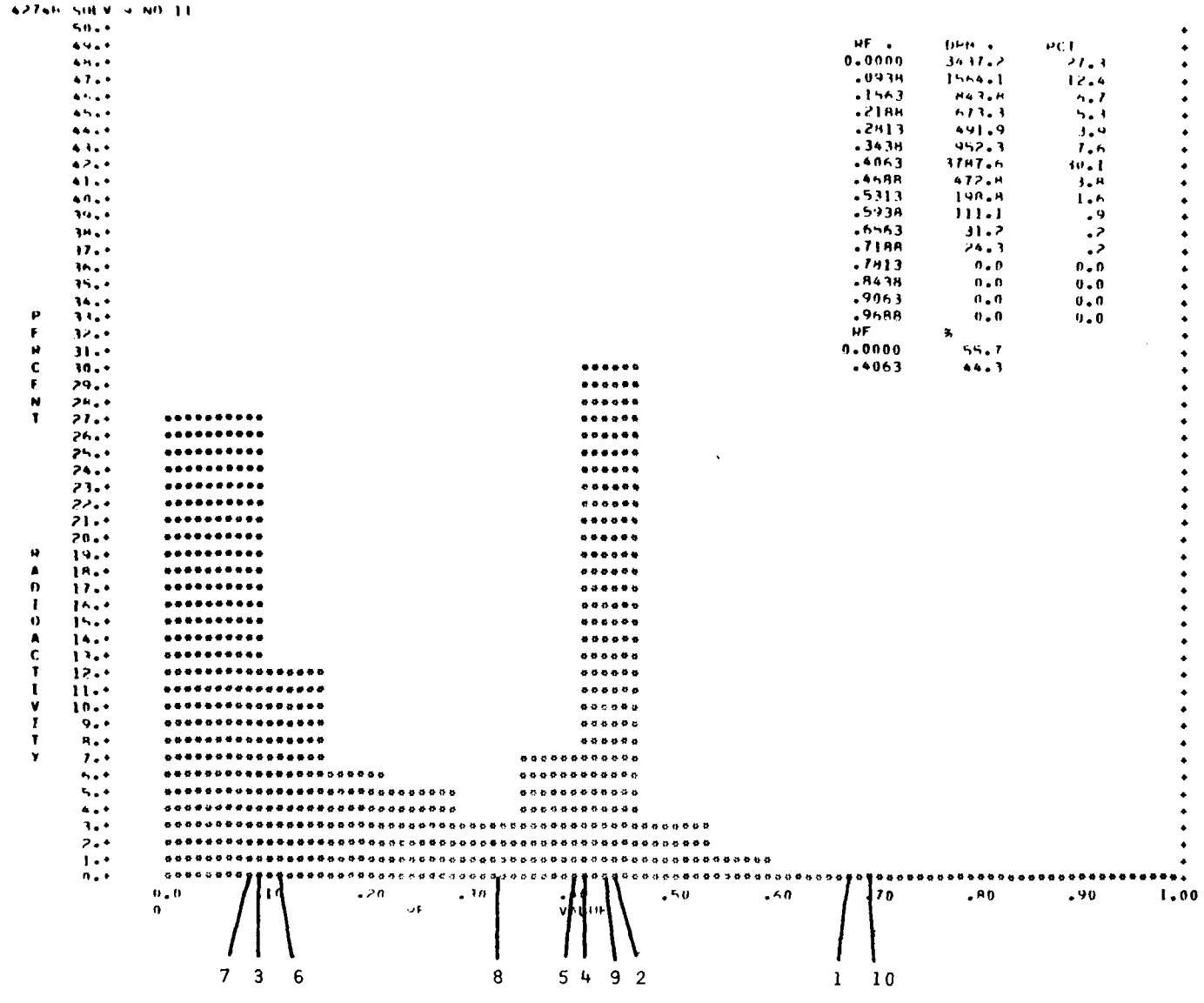

Figure 21-a-IX: Oral Treatment, Incubation with Water, Solvent IX

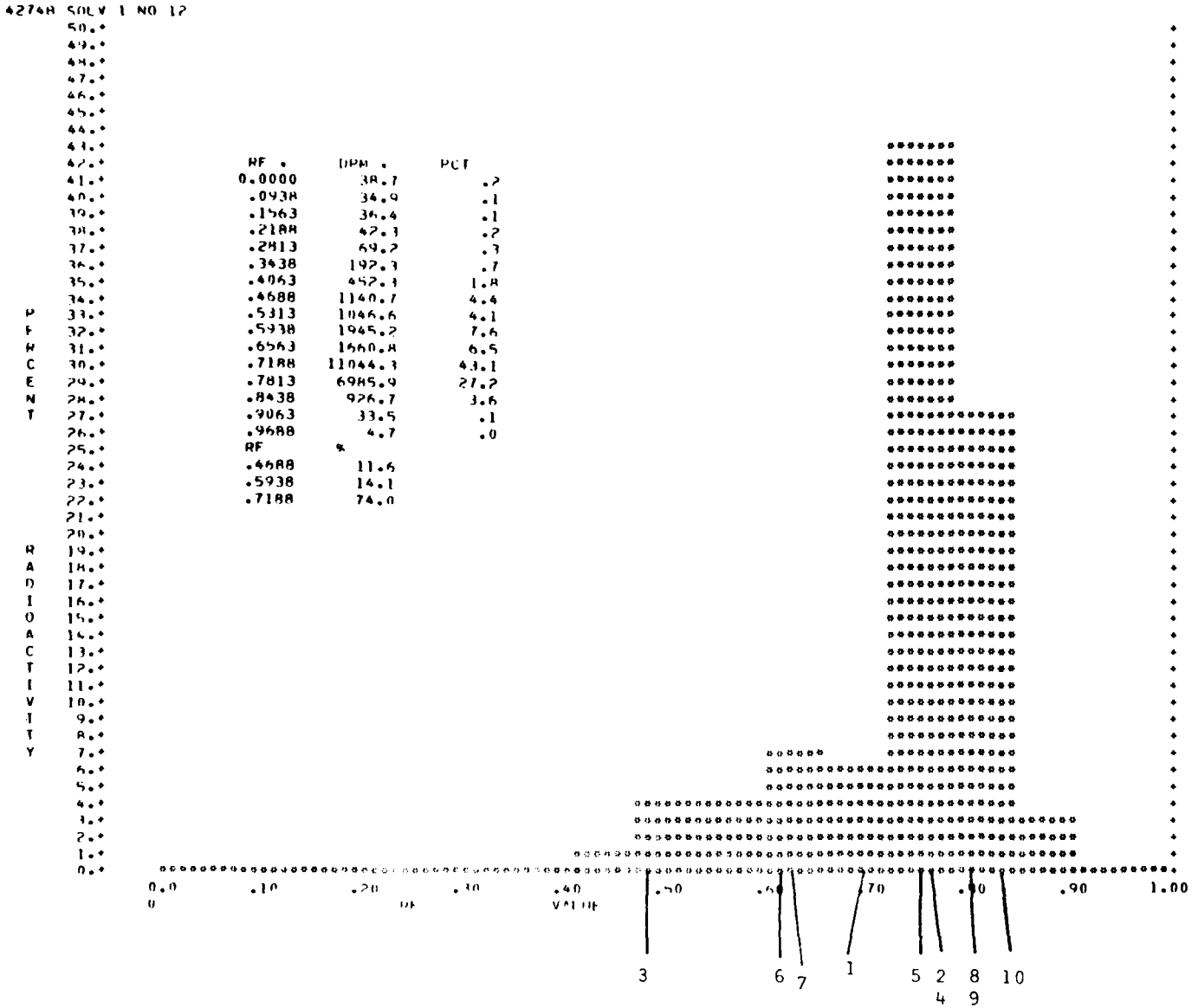

Figure 21-b-I: Oral Treatment, Incubation with β-Glucuronidase, Solvent I

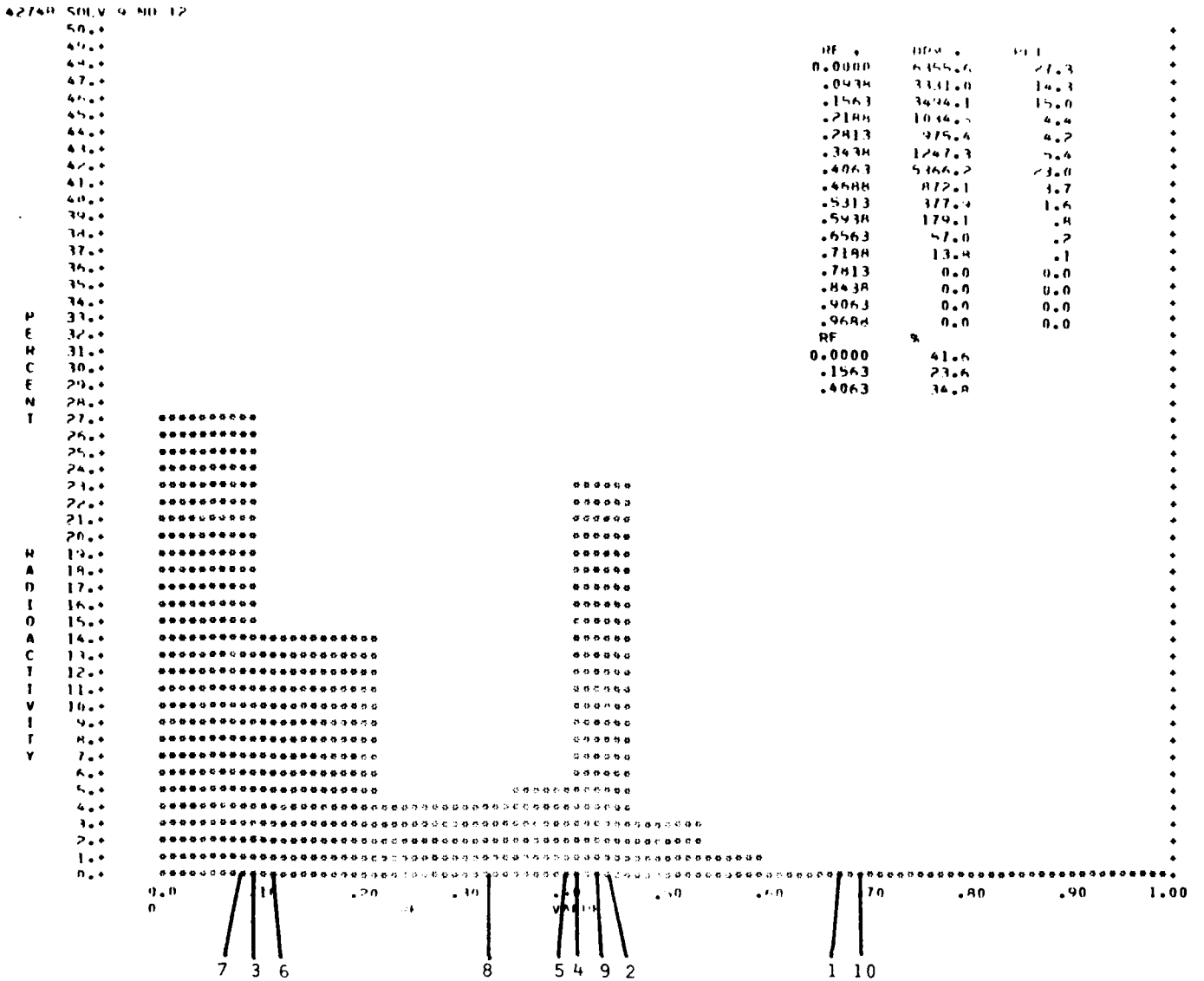

Figure 21-b-IX: Oral Treatment, Incubation with β-Glucuronidase, Solvent IX

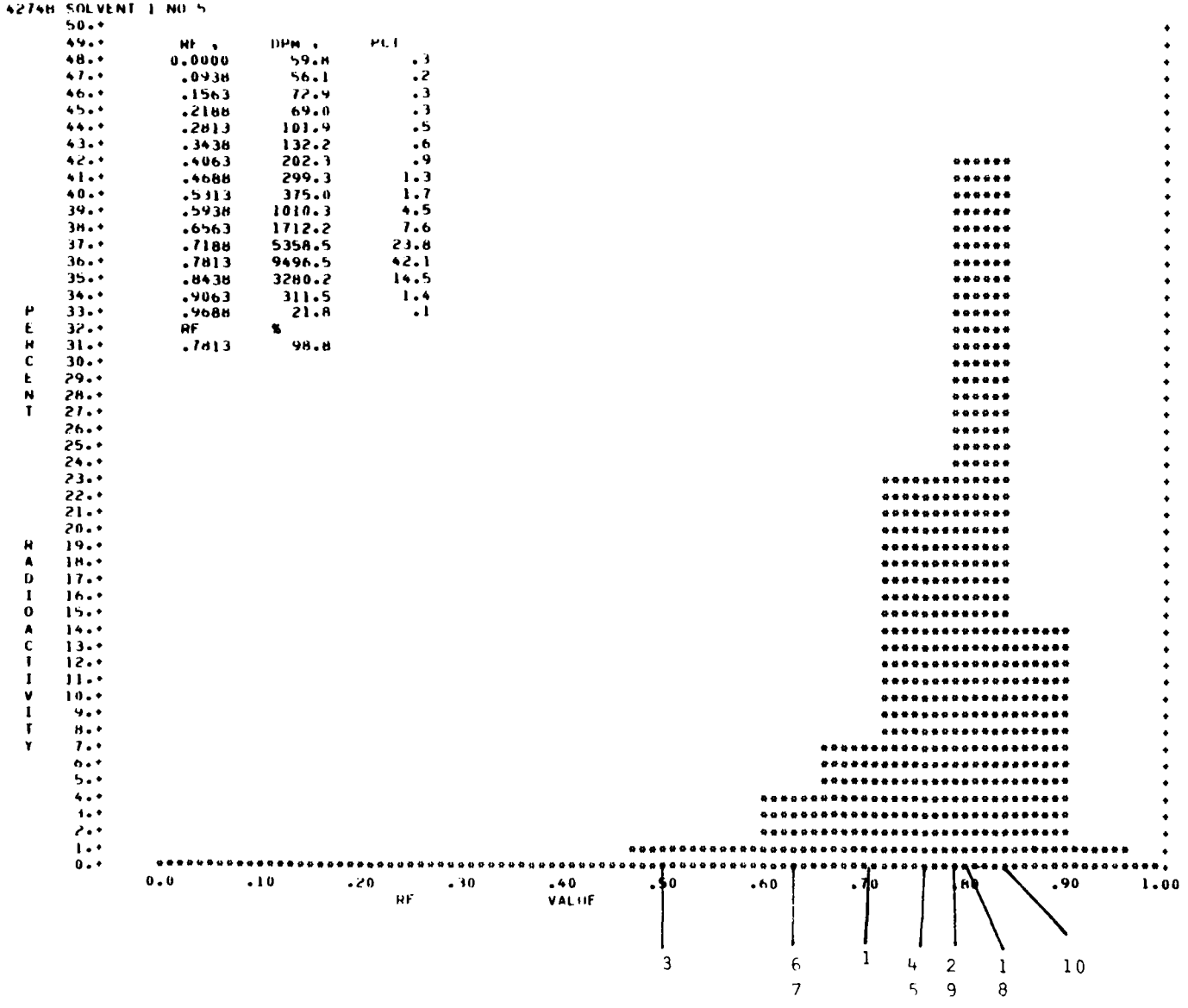

Figure 21-c-I: Dermal Application, Incubation with Water, Solvent I

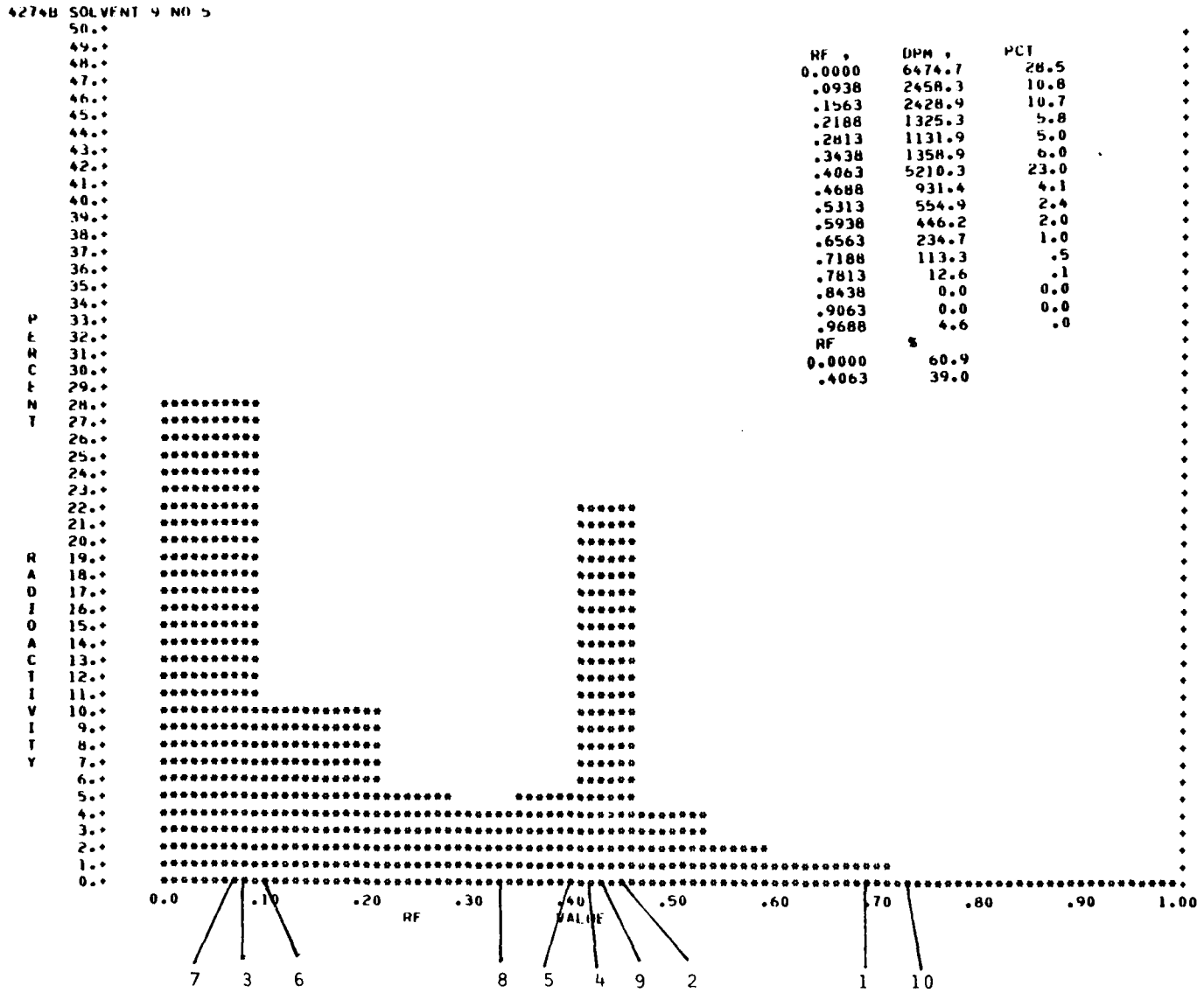

Figure 21-c-IX: Dermal Application, Incubation with Water, Solvent IX

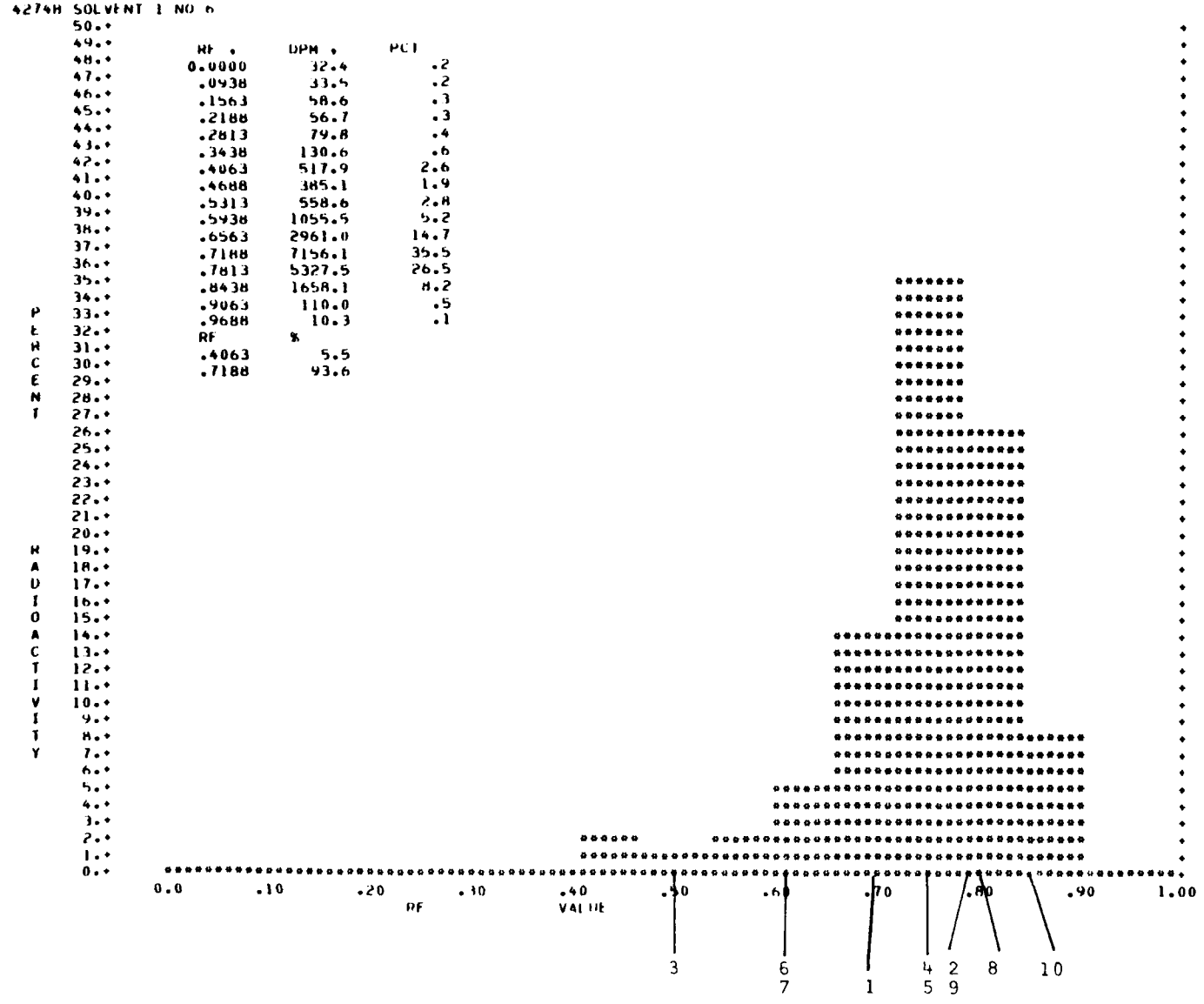

Figure 21-d-I: Dermal Application, Incubation with β-Glucuronidase, Solvent I

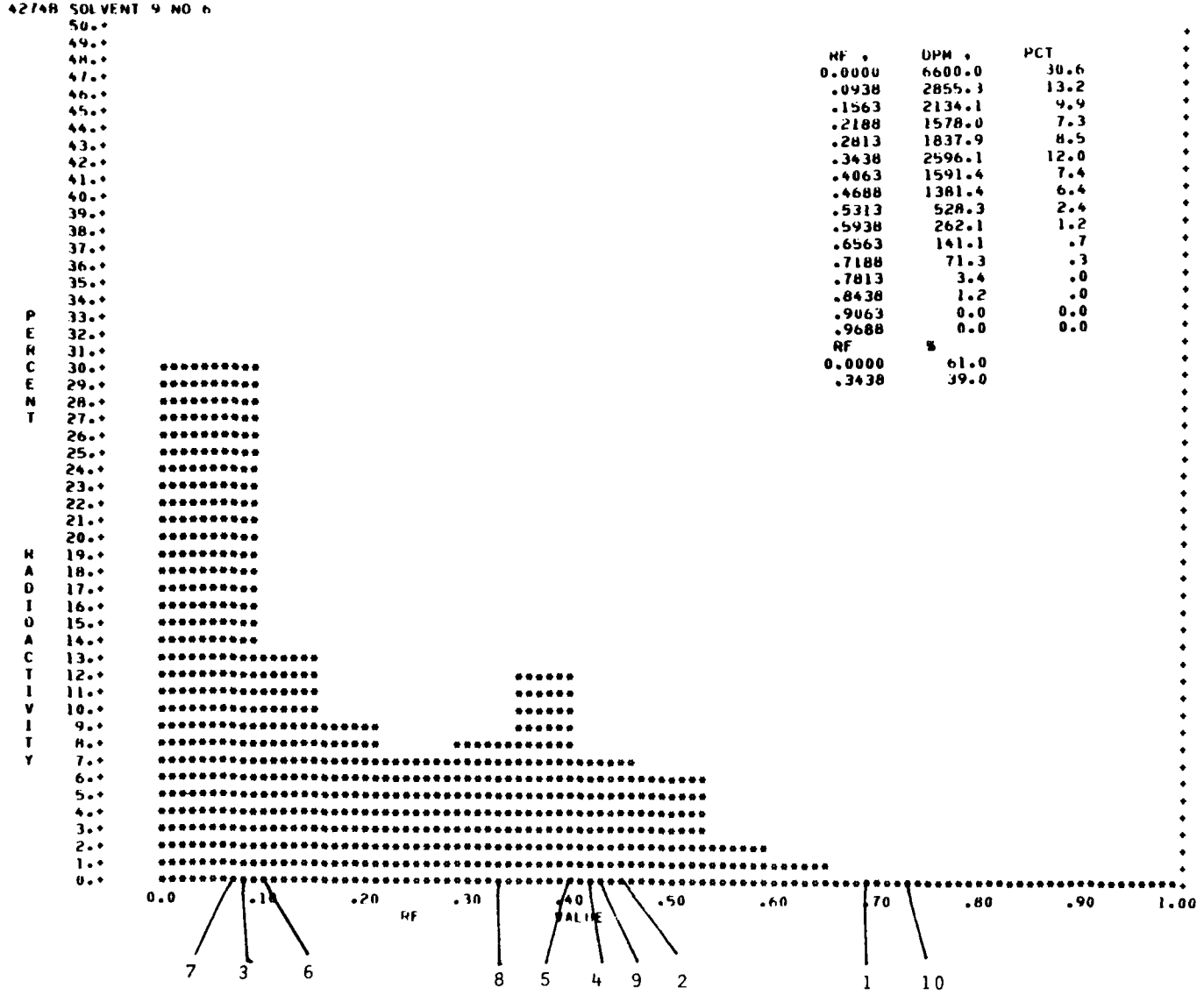

Figure 21-d-IX: Dermal Application, Incubation with β-Glucuronidase, Solvent IX

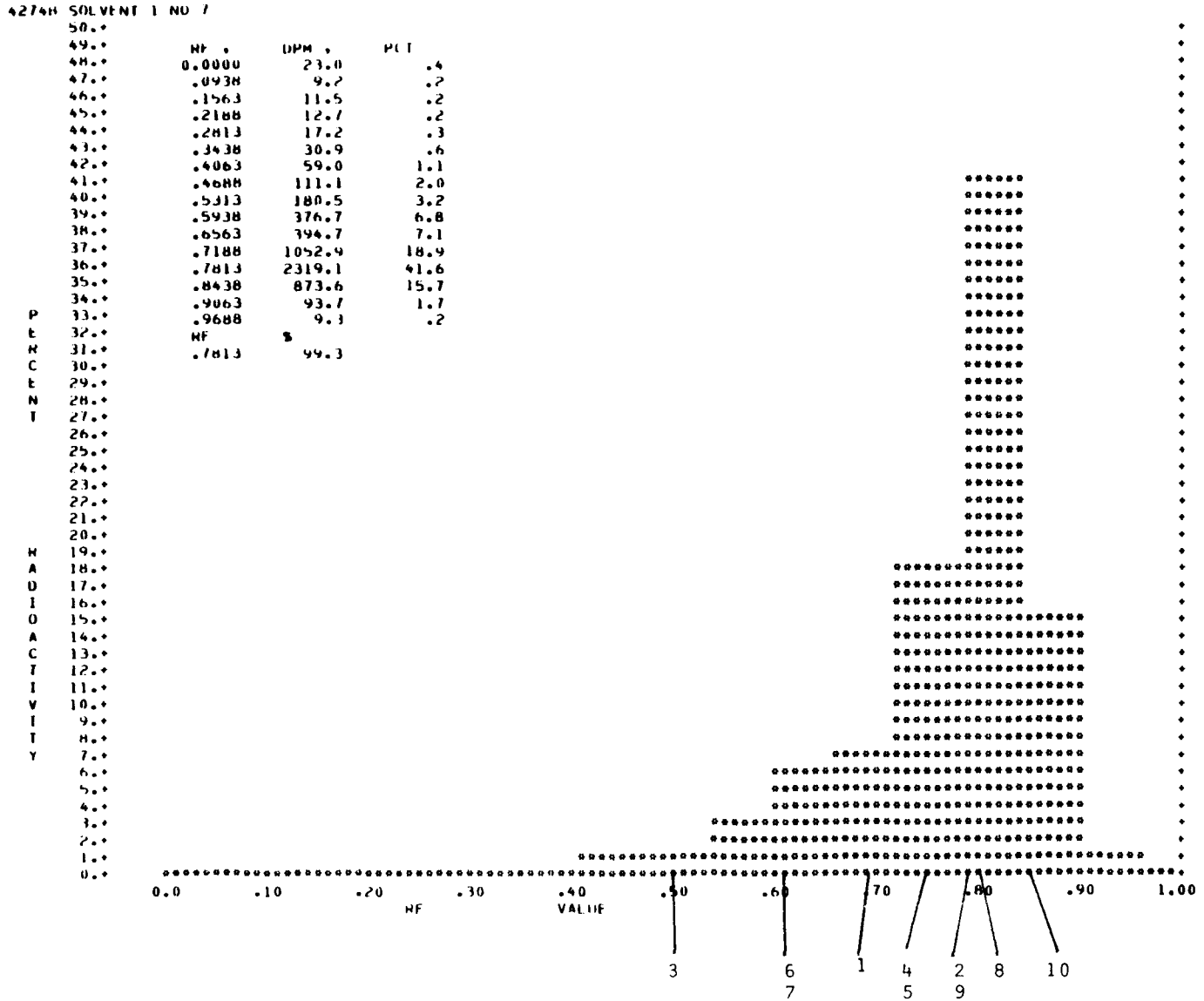

Figure 21-e-I: Oral Treatment, Incubation with Water, Solvent I

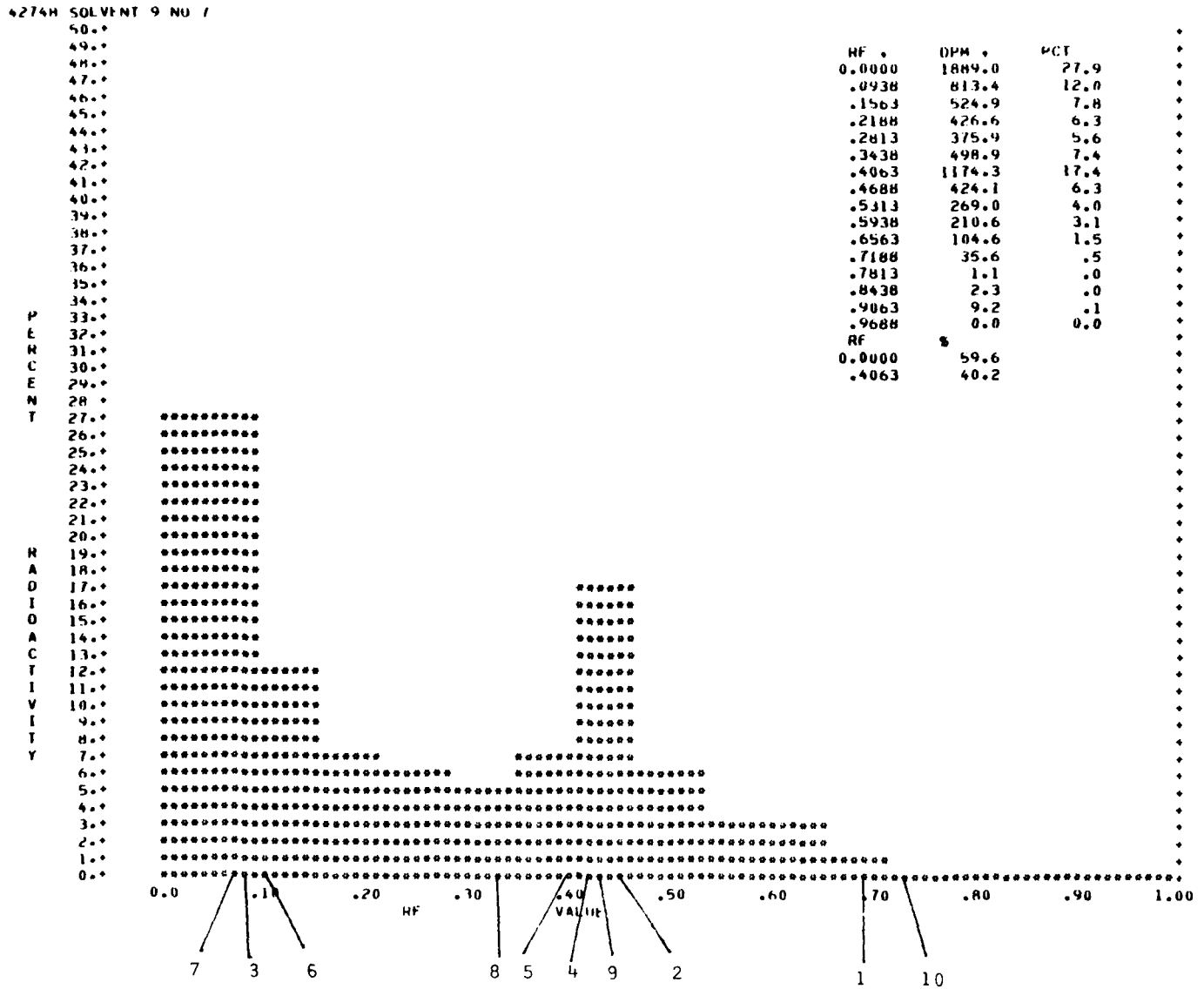

Figure 21-e-IX: Oral Treatment, Incubation with Water, Solvent IX

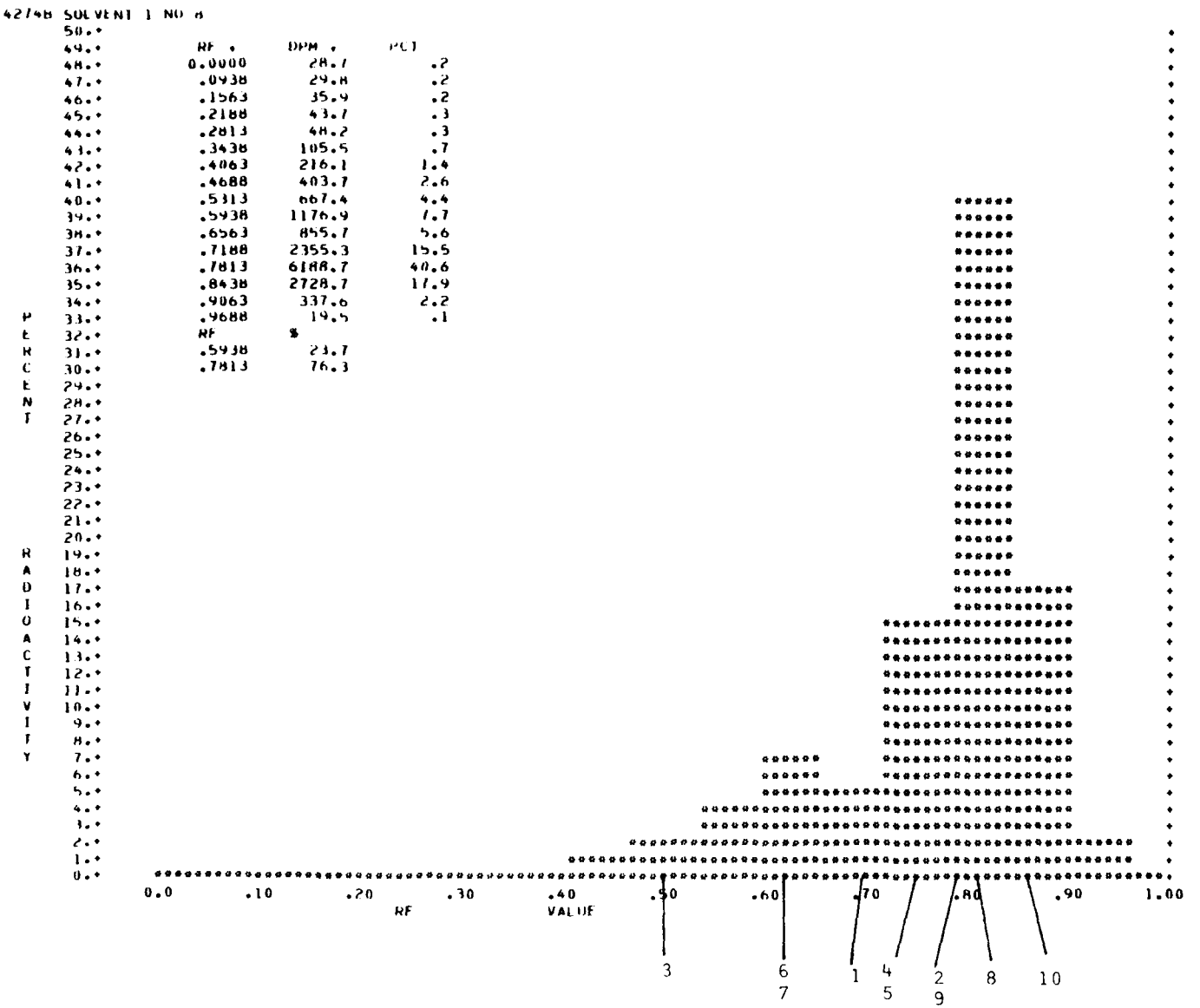

Figure 21-f-I: Oral Treatment, Incubation with β-Glucuronidase, Solvent I

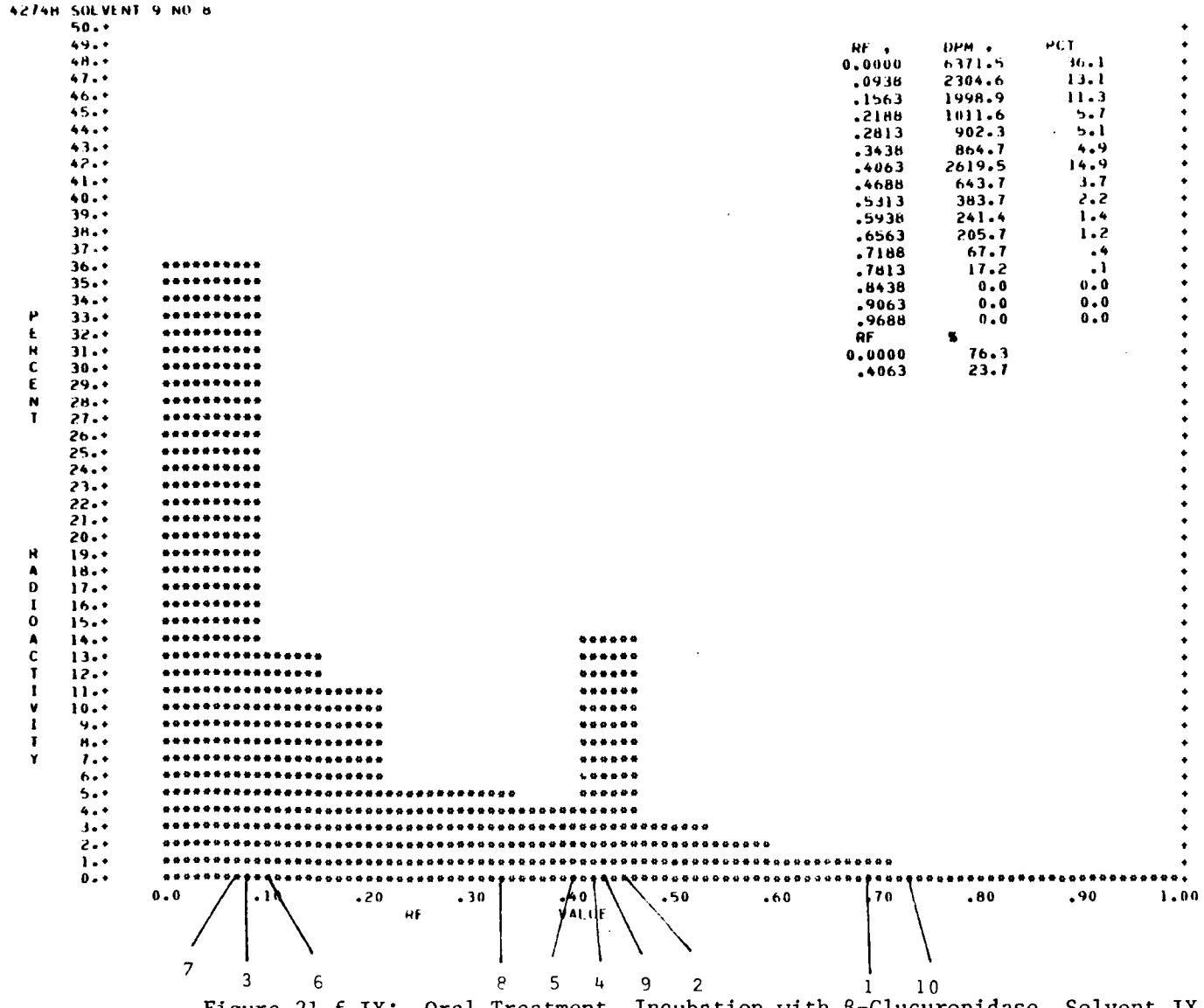

Figure 21-f-IX: Oral Treatment, Incubation with β-Glucuronidase, Solvent IX

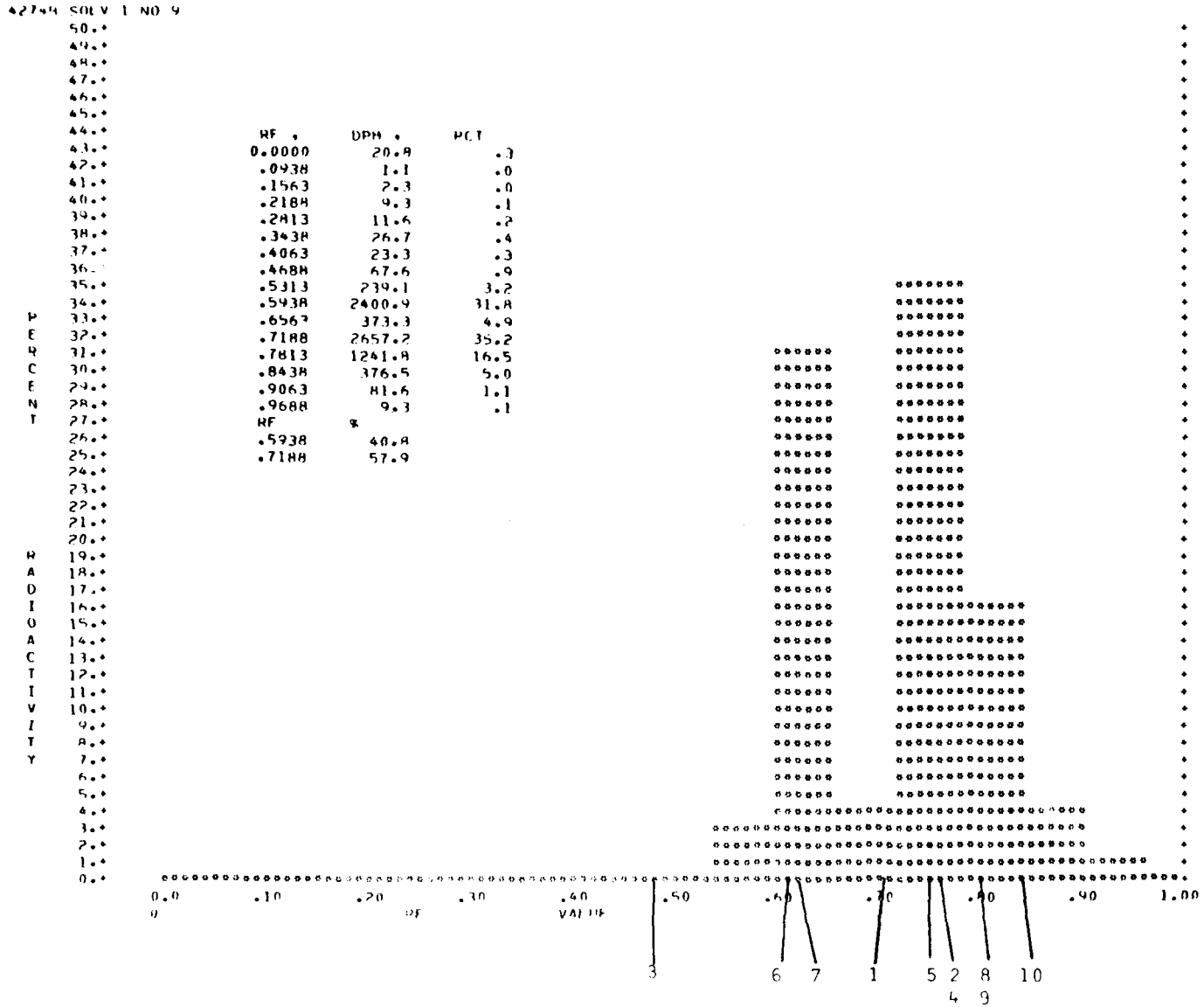

Figure 21-g-I: Dermal Application, Incubation with Water Solvent I

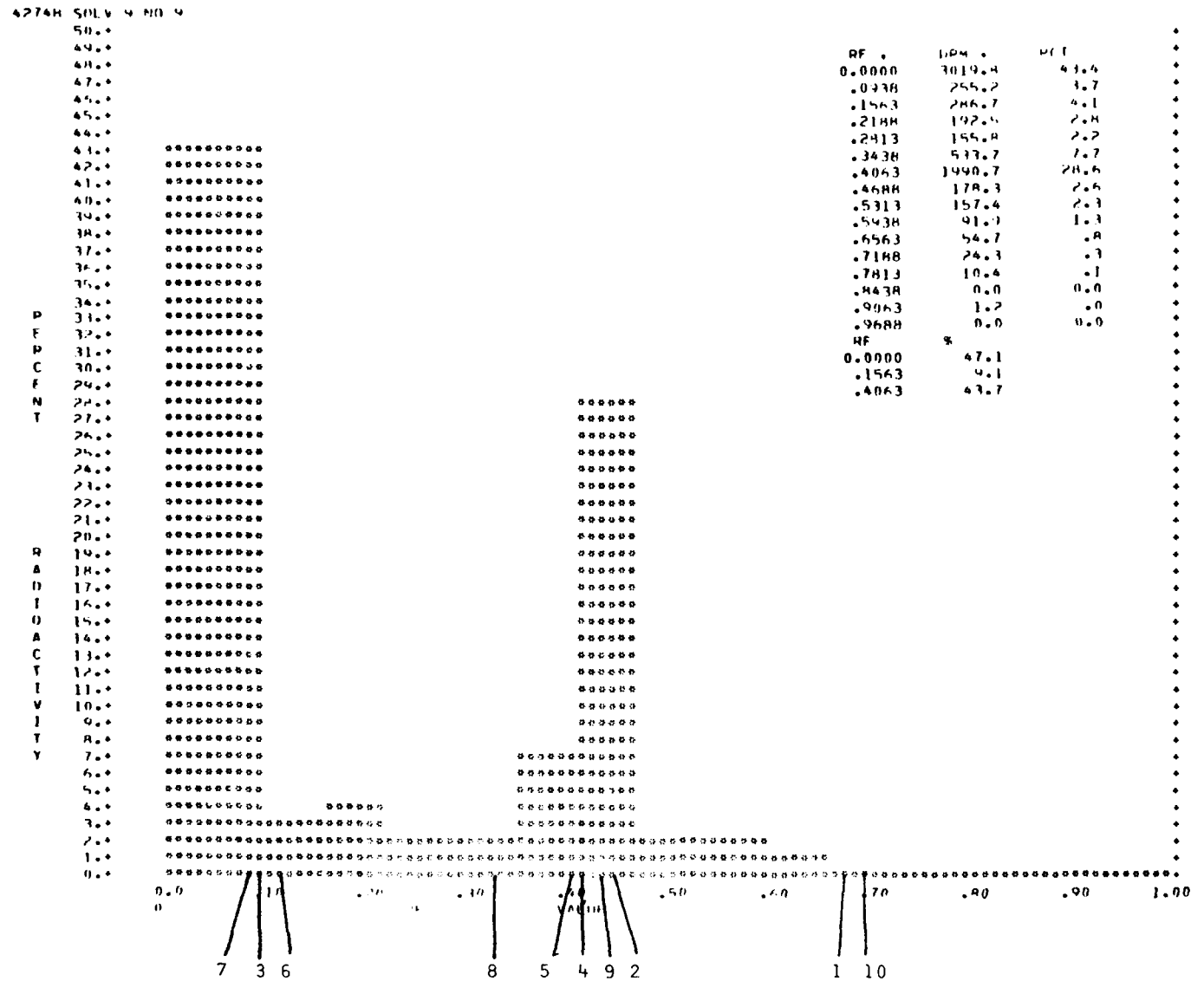

Figure 21-g-IX: Dermal Application, Incubation with Water, Solvent IX

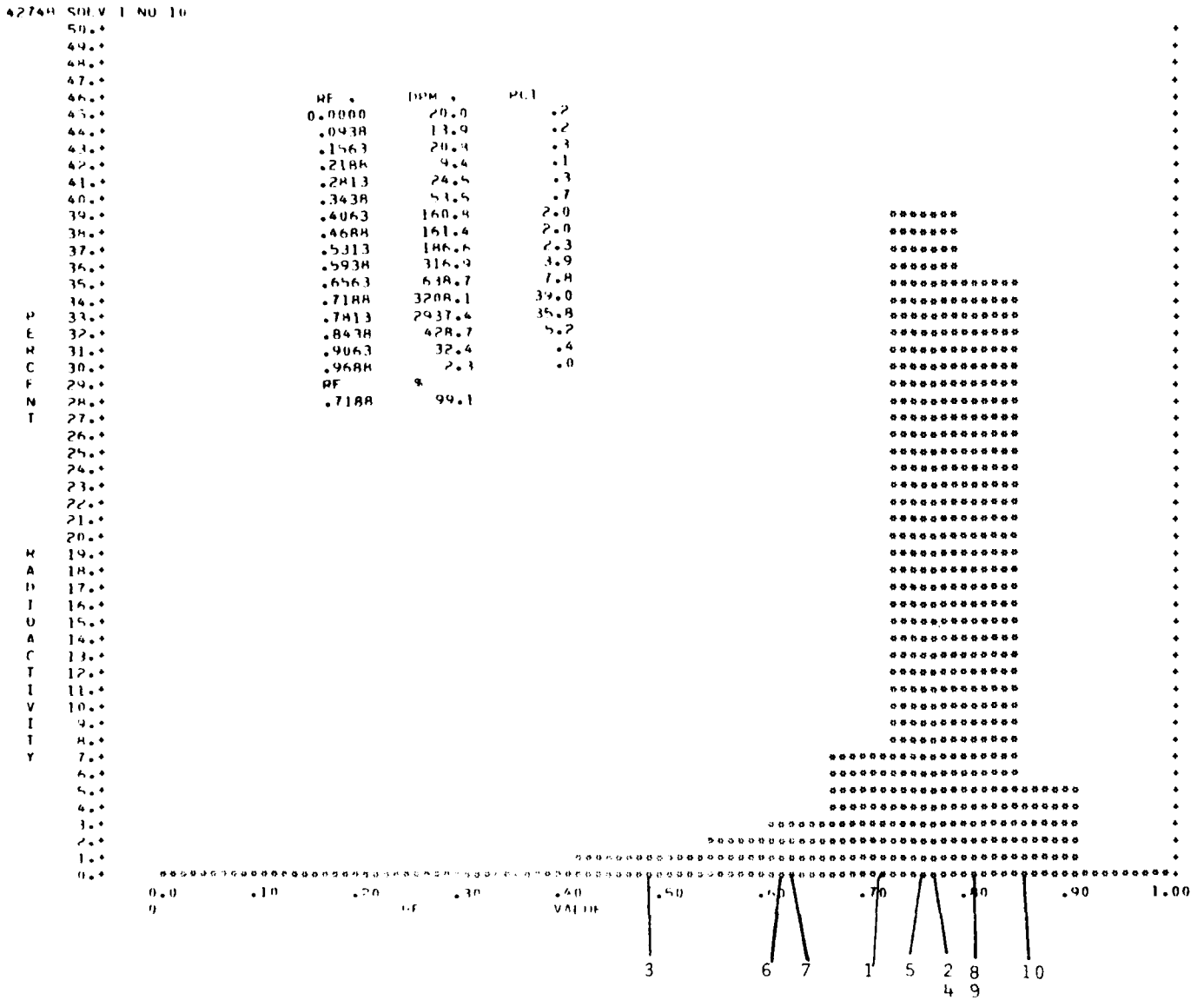

Figure 21-h-I: Dermal Application, Incubation with β-Glucuronidase, Solvent I

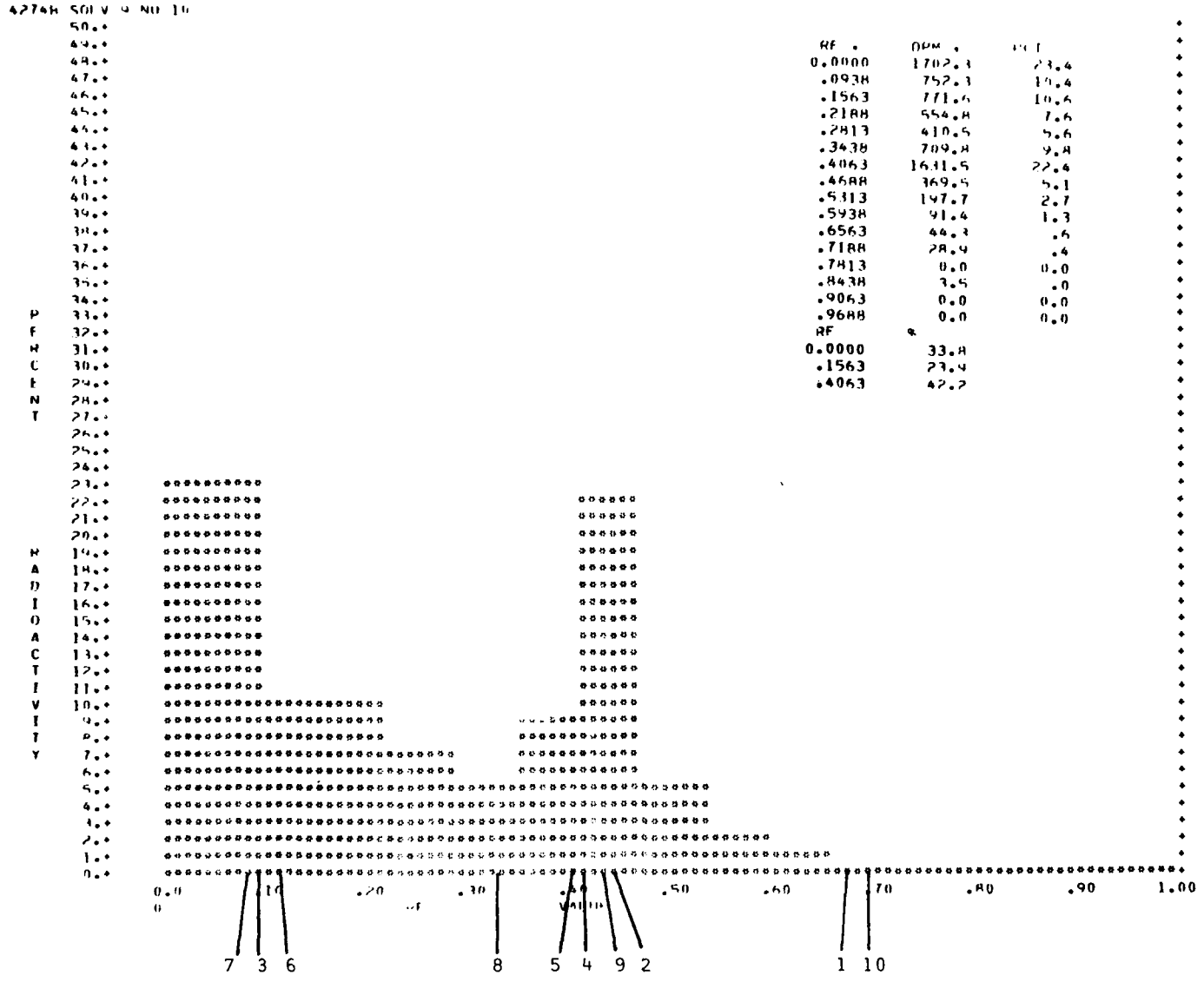

Figure 21-h-IX: Dermal Application, Incubation with β-Glucuronidase, Solvent IX

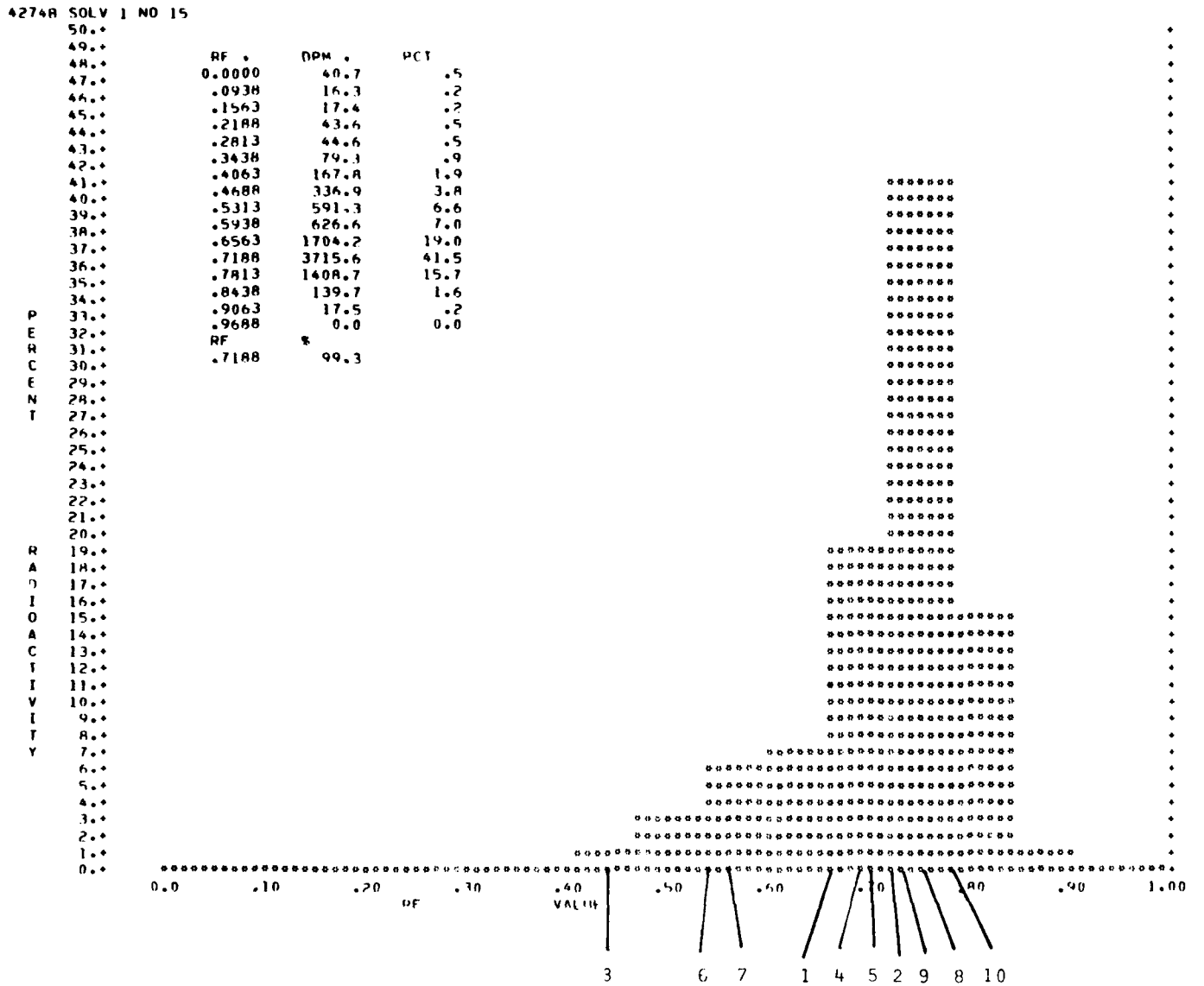

Figure 21-k-I: Oral Treatment, Incubation with Water, Solvent I

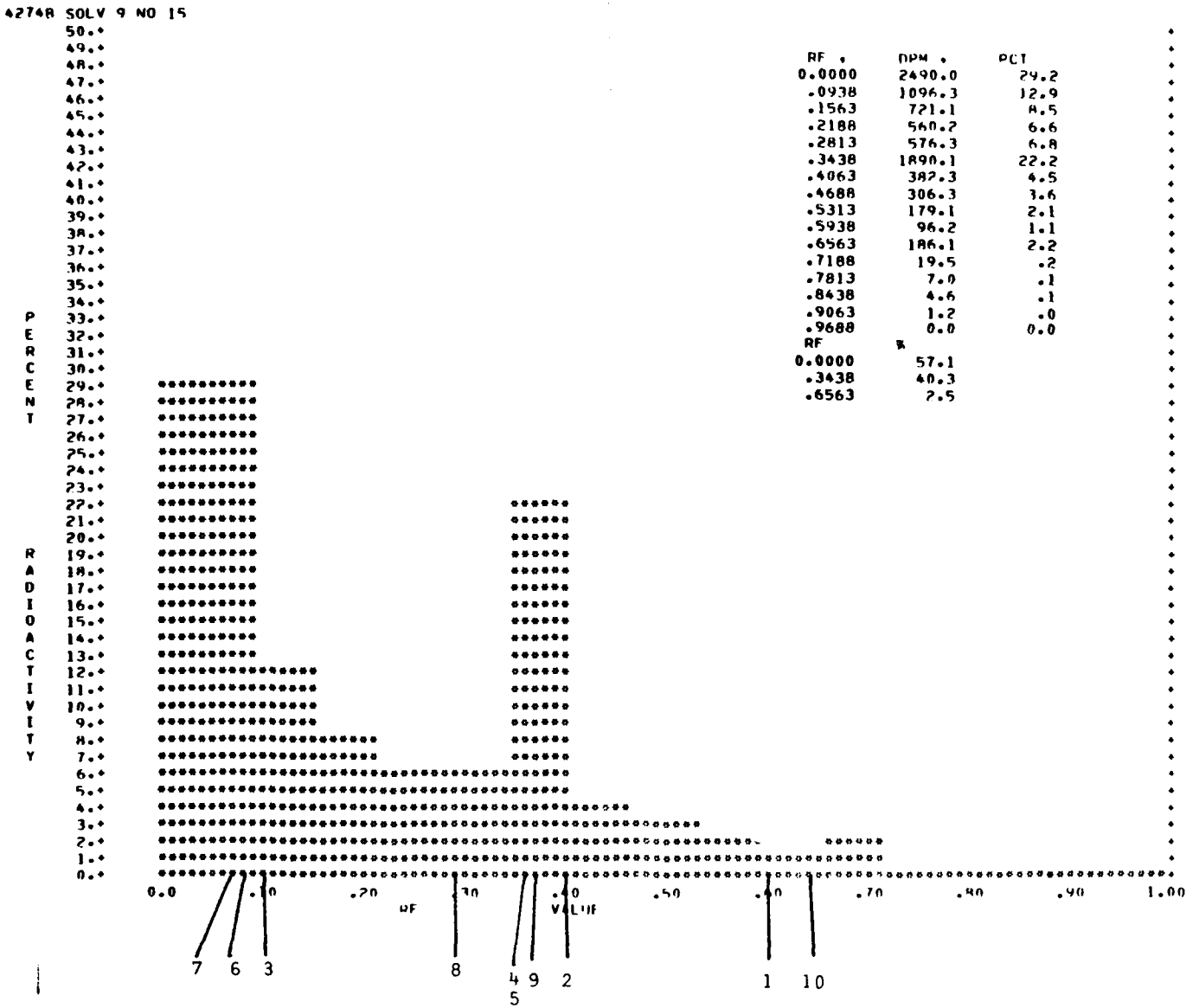

Figure 21-k-IX: Oral Treatment, Incubation with Water, Solvent IX

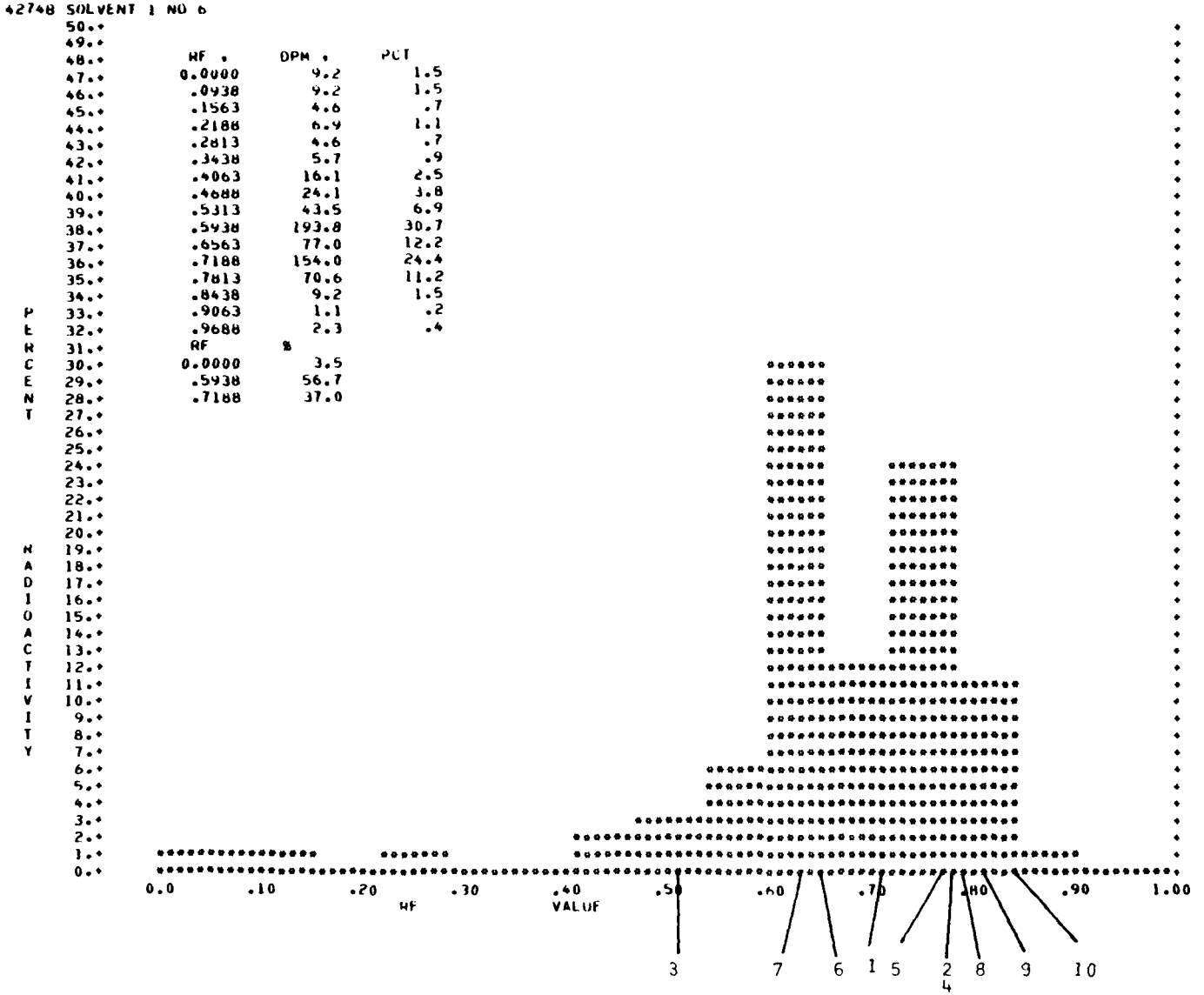

Figure 21-1-I: Oral Treatment, Incubation with β-Glucuronidase, Solvent I

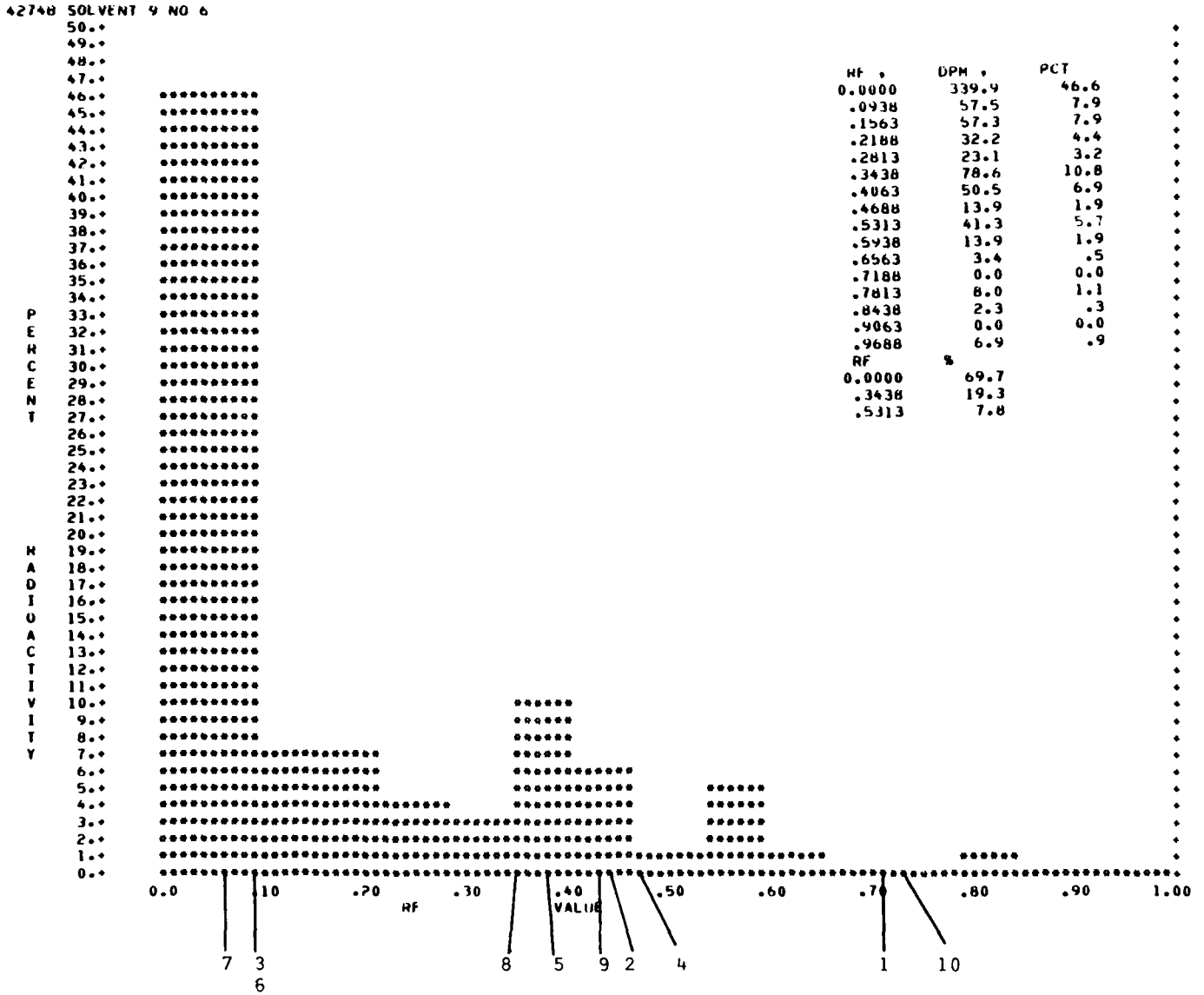

Figure 21-1-IX: Oral Treatment, Incubation with β-Glucuronidase, Solvent IX

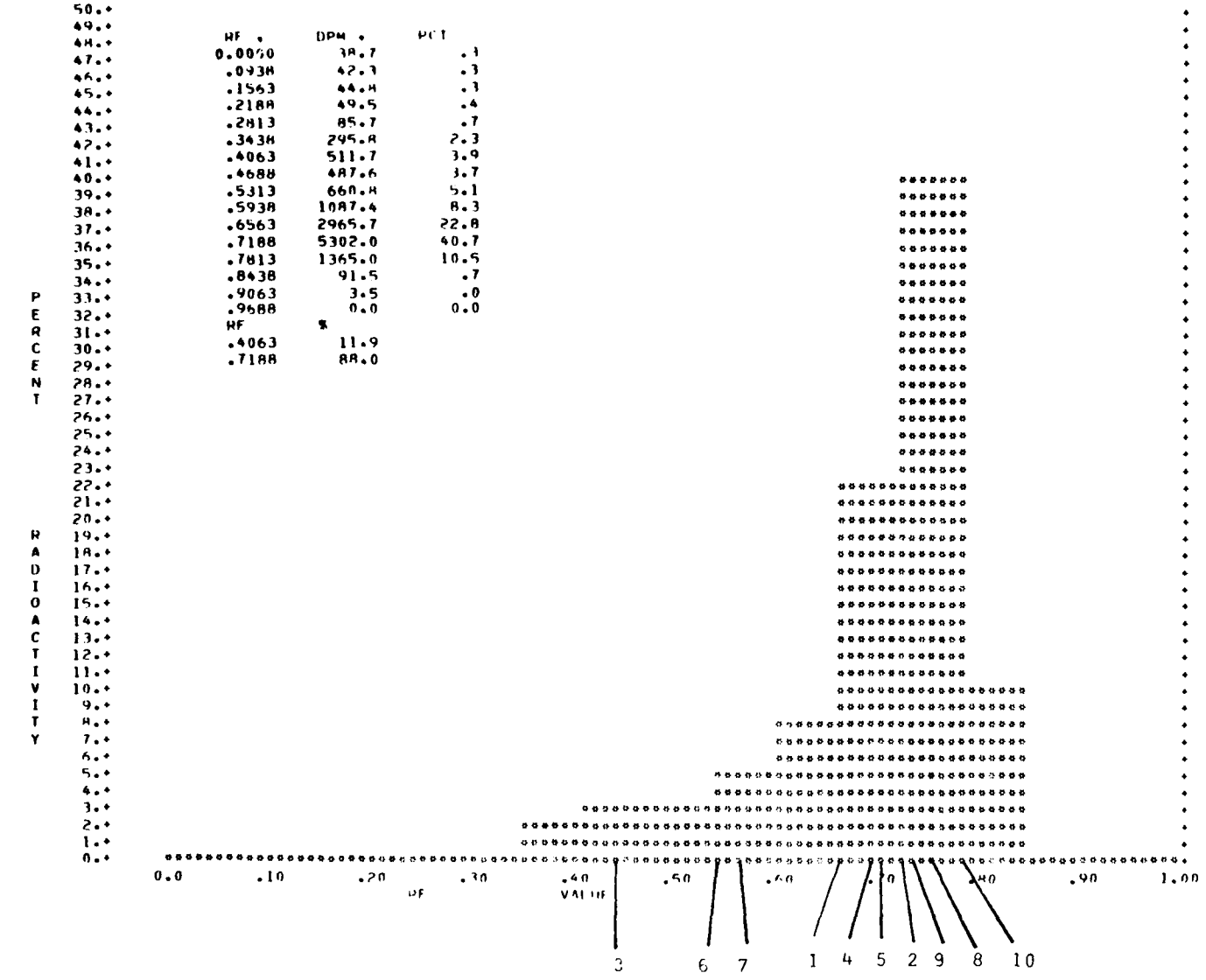

Figure 21-m-I: Dermal Application, Incubation with Water, Solvent I

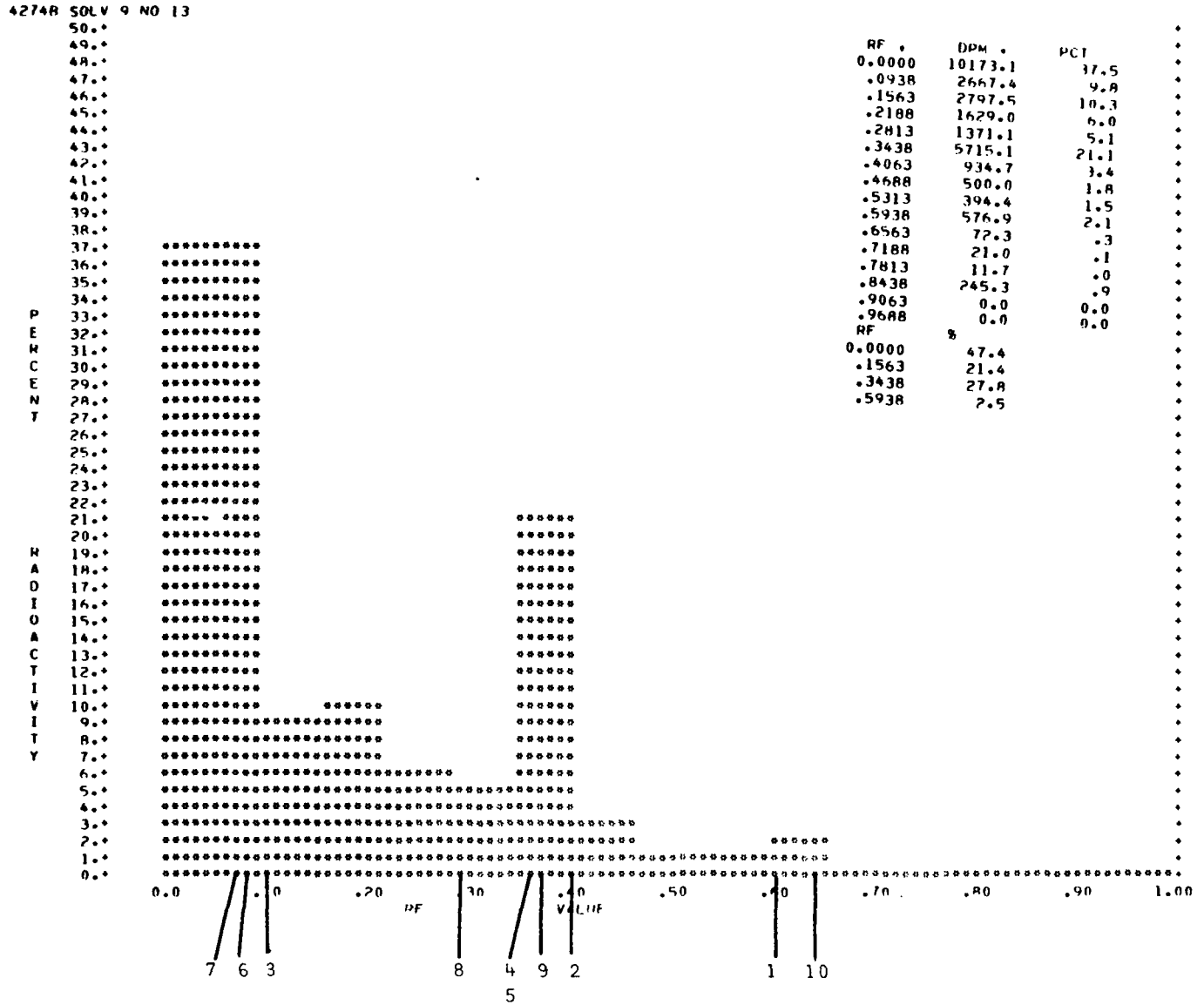

Figure 21-m-IX: Dermal Application, Incubation with Water, Solvent IX

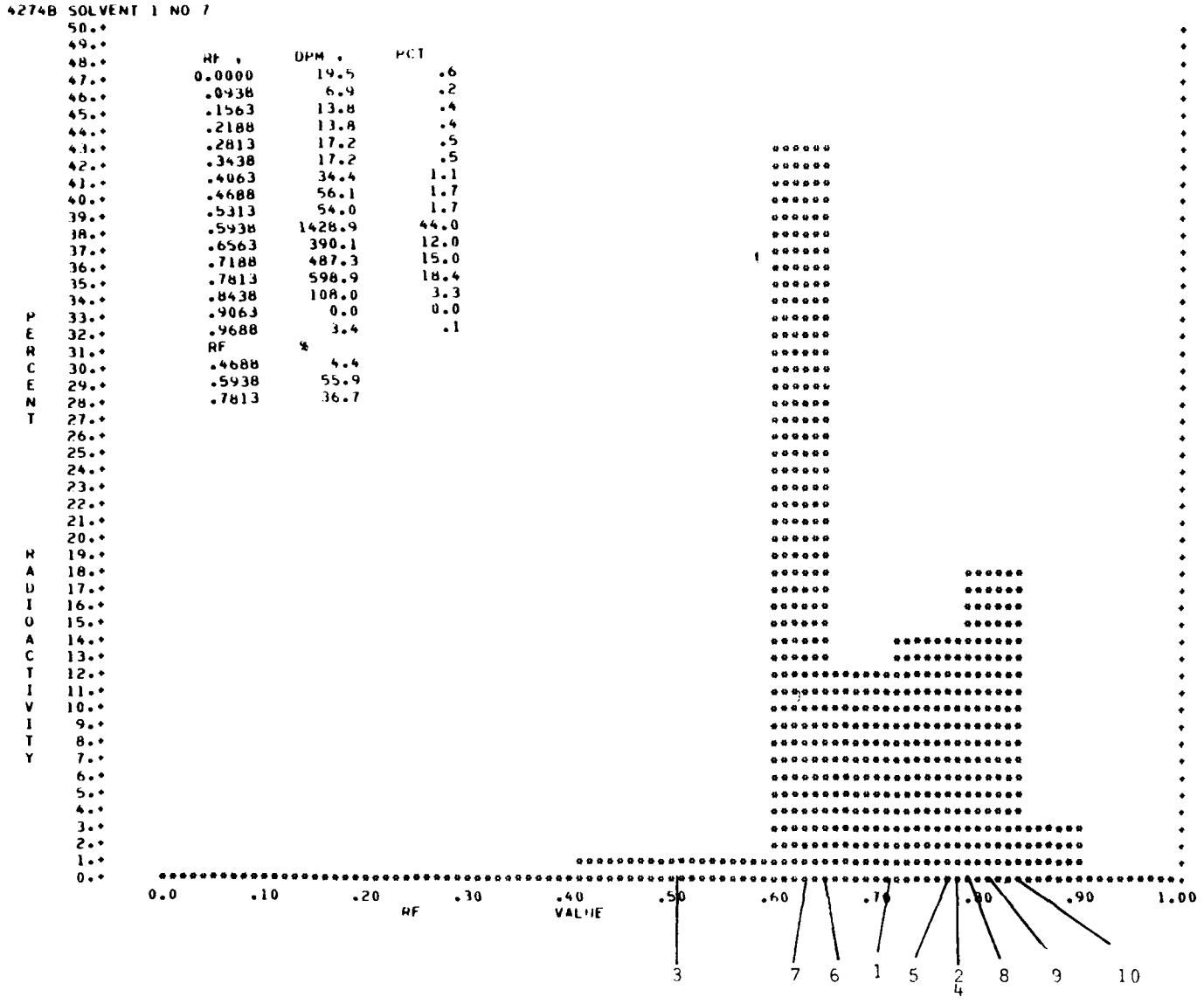

Figure 21-n-I: Dermal Application, Incubation with β-Glucuronidase, Solvent I

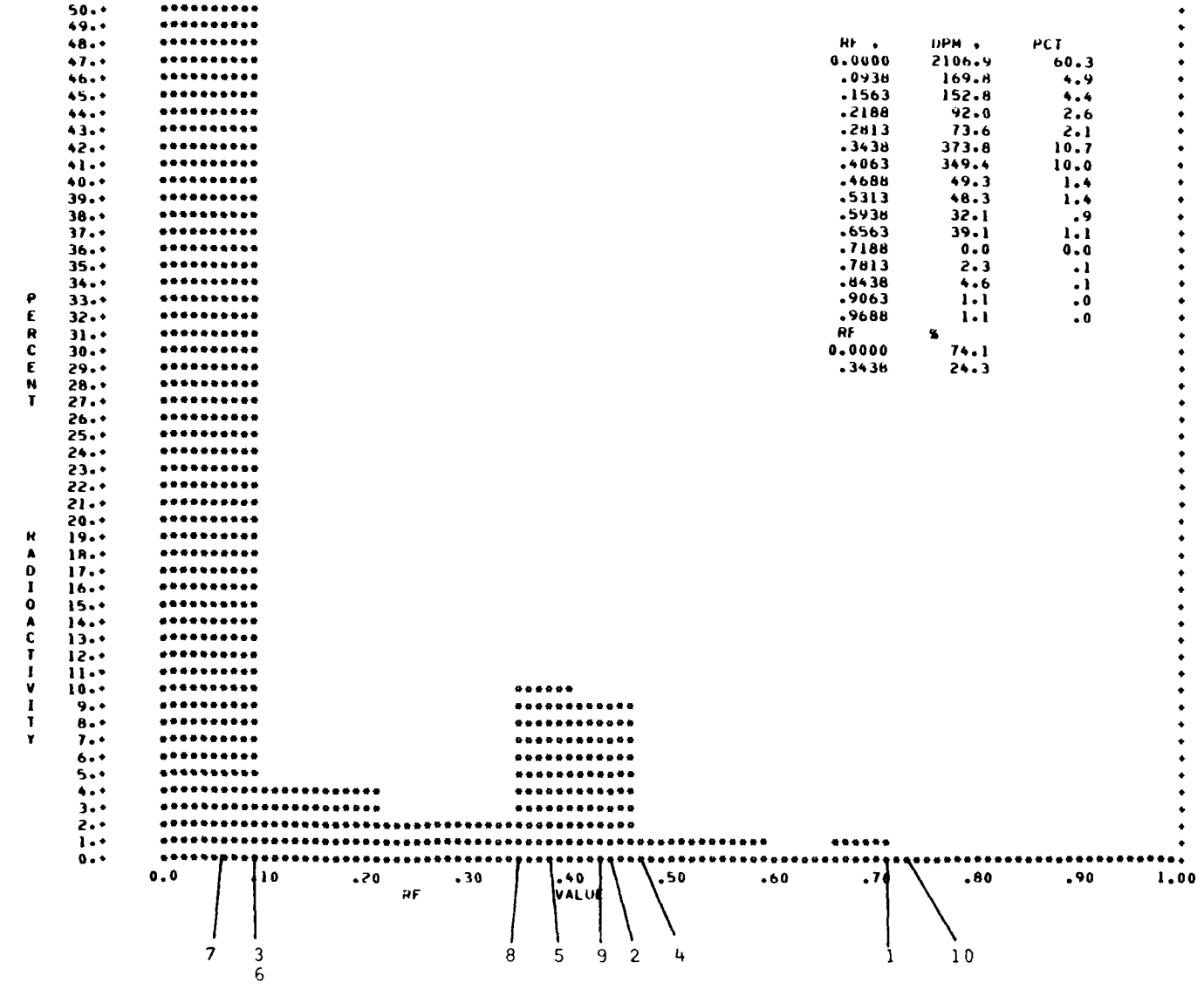

Figure 21-n-IX: Dermal Application, Incubation with β-Glucuronidase, Solvent IX

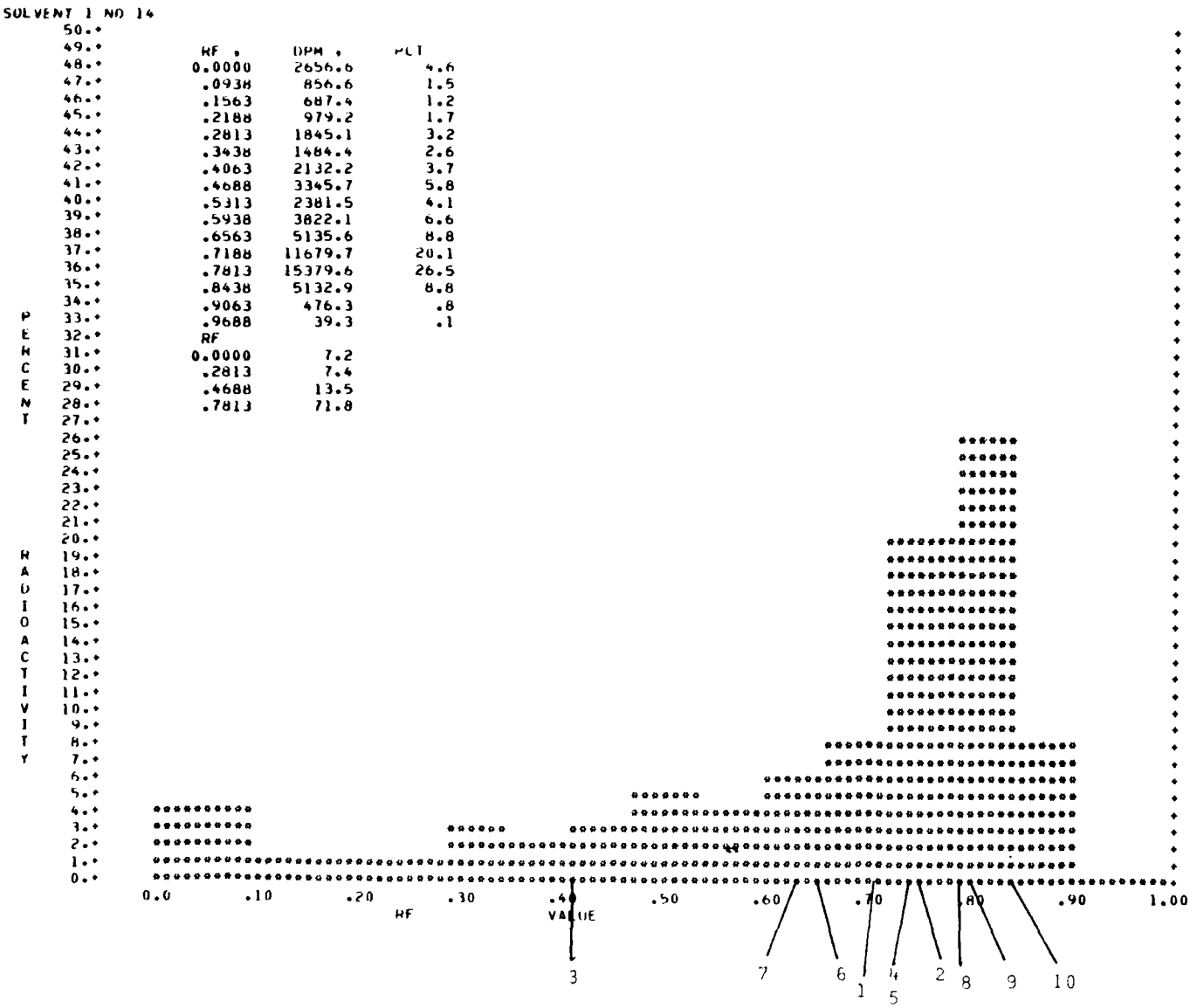

Figure 21-o-I: Oral Treatment, Incubation with β-Glucuronidase, Solvent I

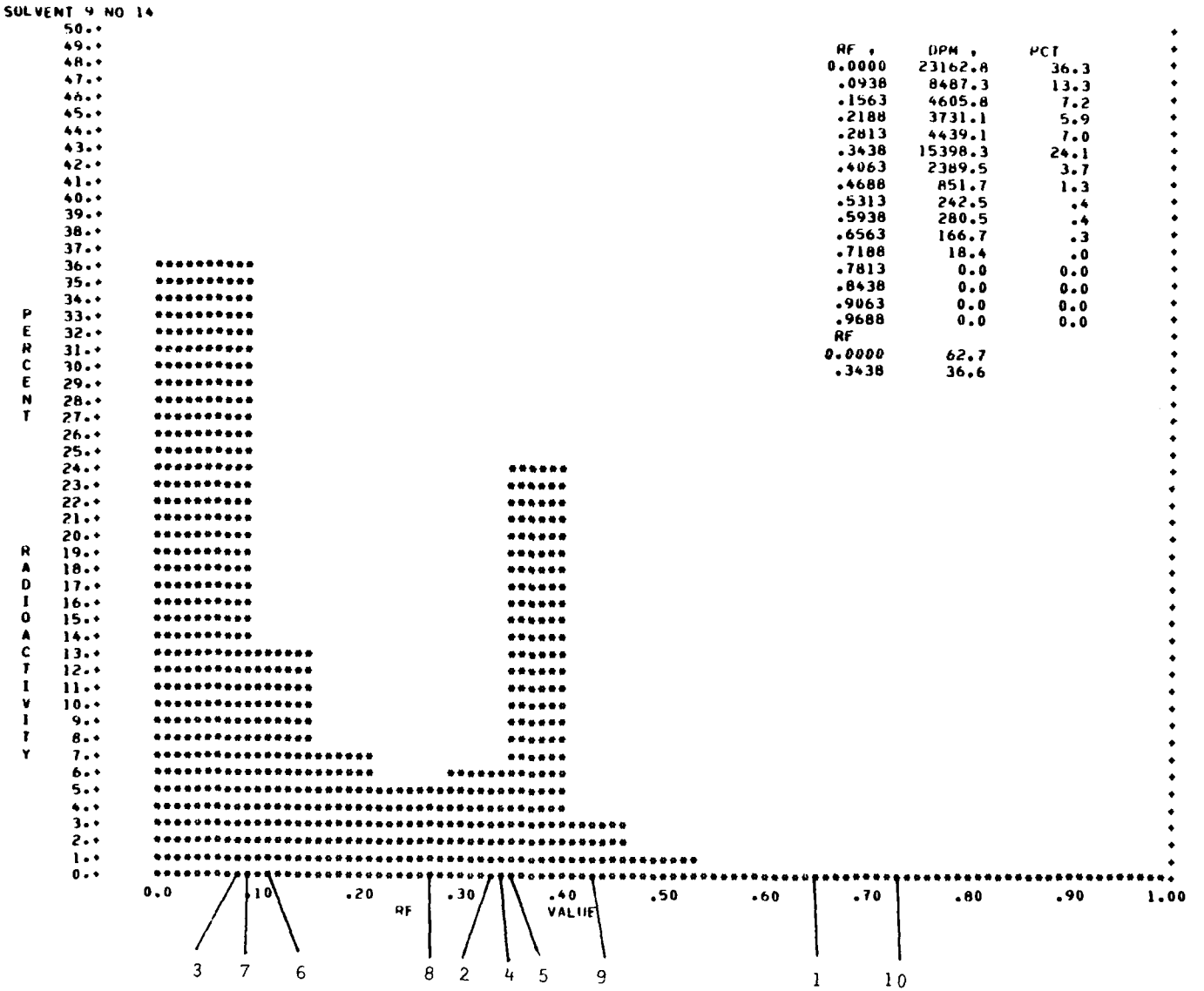

Figure 21-o-IX: Oral Treatment, Incubation with β-Glucuronidase, Solvent IX

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SPECIES DIFFERENCES IN THE DISPOSITION AND METABOLISM OF 2,4,6--ETC(U)
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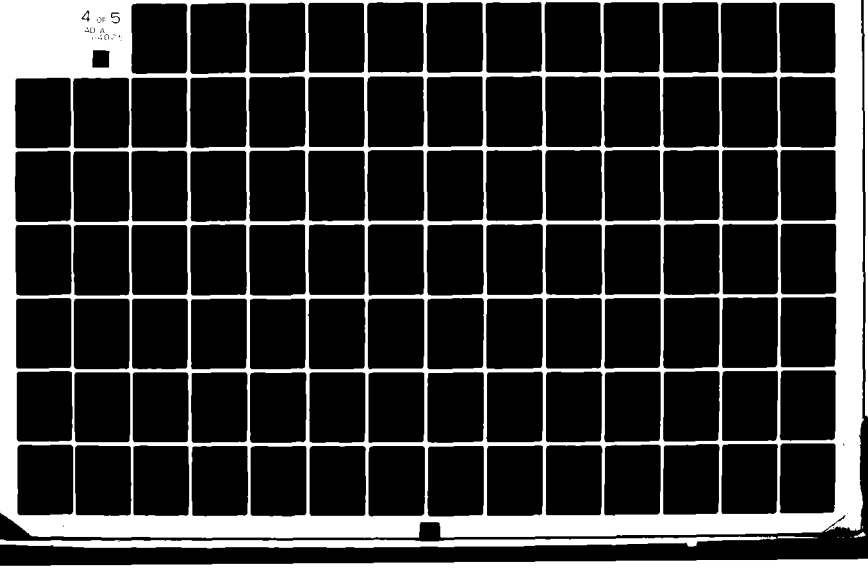


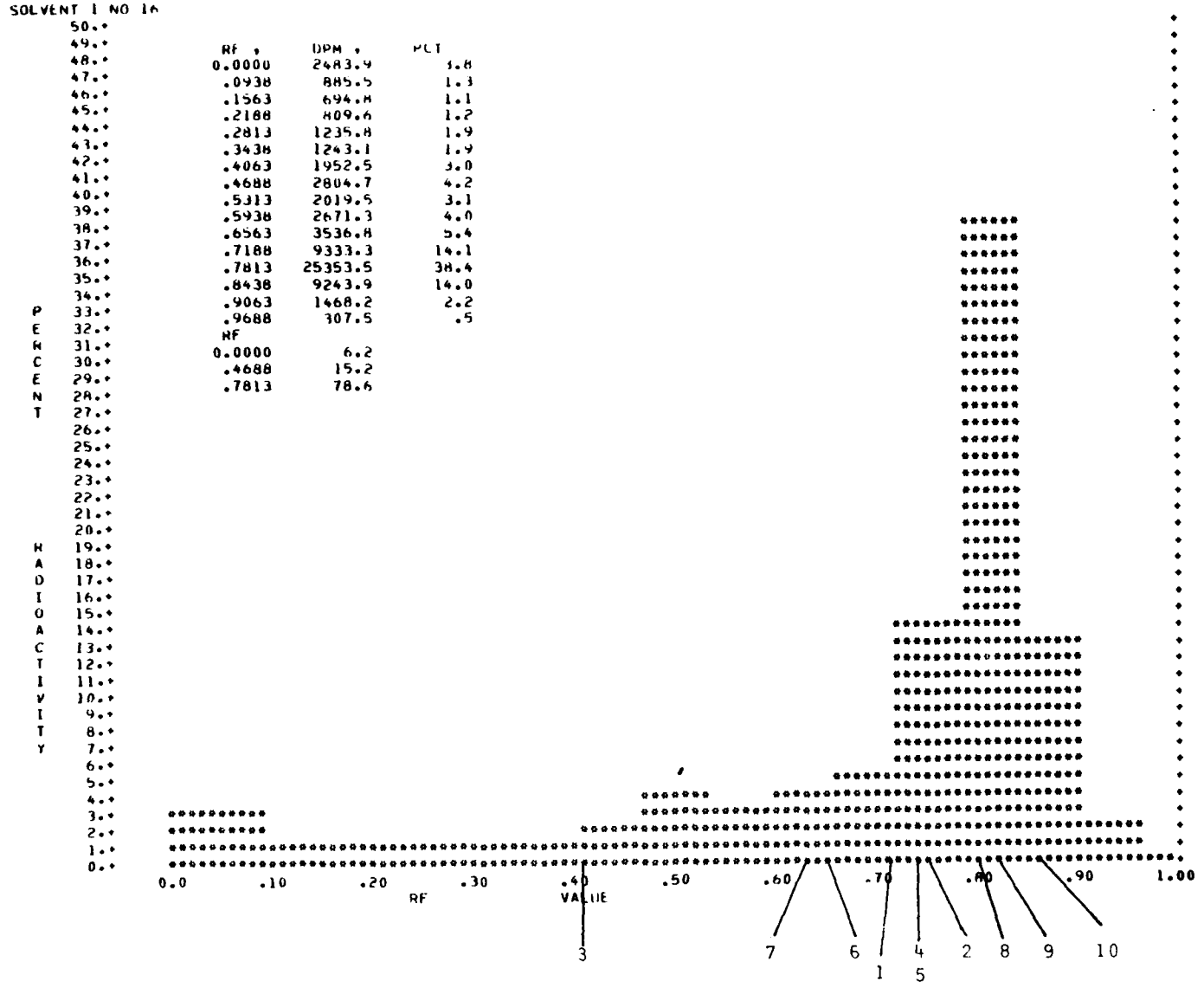


Figure 21-p-I: Dermal Application, Incubation with β-Glucuronidase, Solvent I

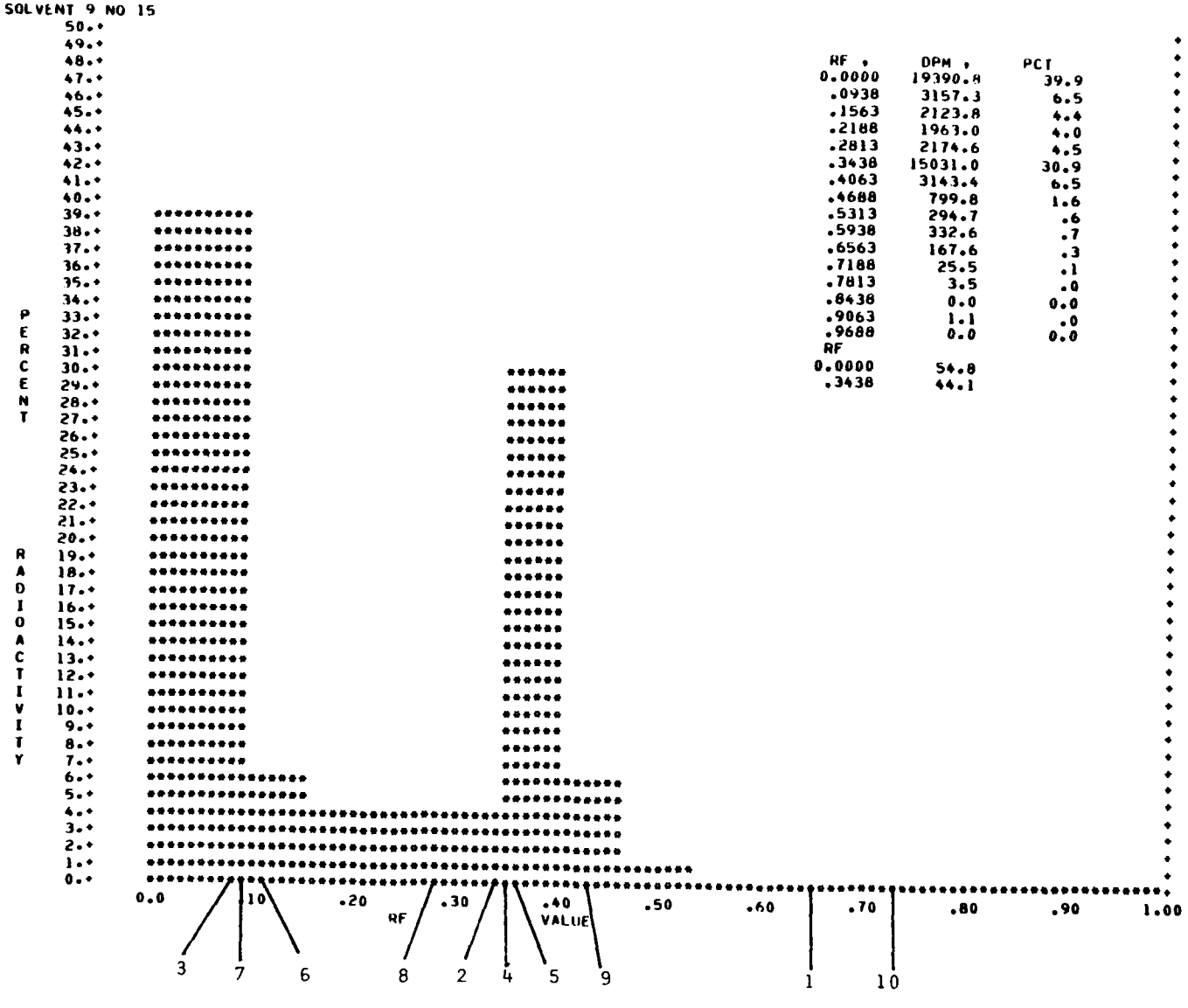


Figure 21-p-IX: Dermal Application, Incubation with β-Glucuronidase, Solvent IX

Figure 22: TLC of Ethyl Acetate-Extractable Products Obtained from 24-Hr Urine of Male Dogs Treated Orally or Dermally with ^{14}C-TNT. Samples of urine were incubated with acetate buffer and β-glucuronidase before extraction with ethyl acetate. Incubation with acetate buffer and water served as control. Reference standards are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzylalcohol
3. Trinitrobenzoic Acid
4. 4-Amino-2,6-Dinitrotoluene
5. 2-Amino-4,6-Dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2,6'-Tetranitro-4,4'-azoxytoluene

Figure 22 follows

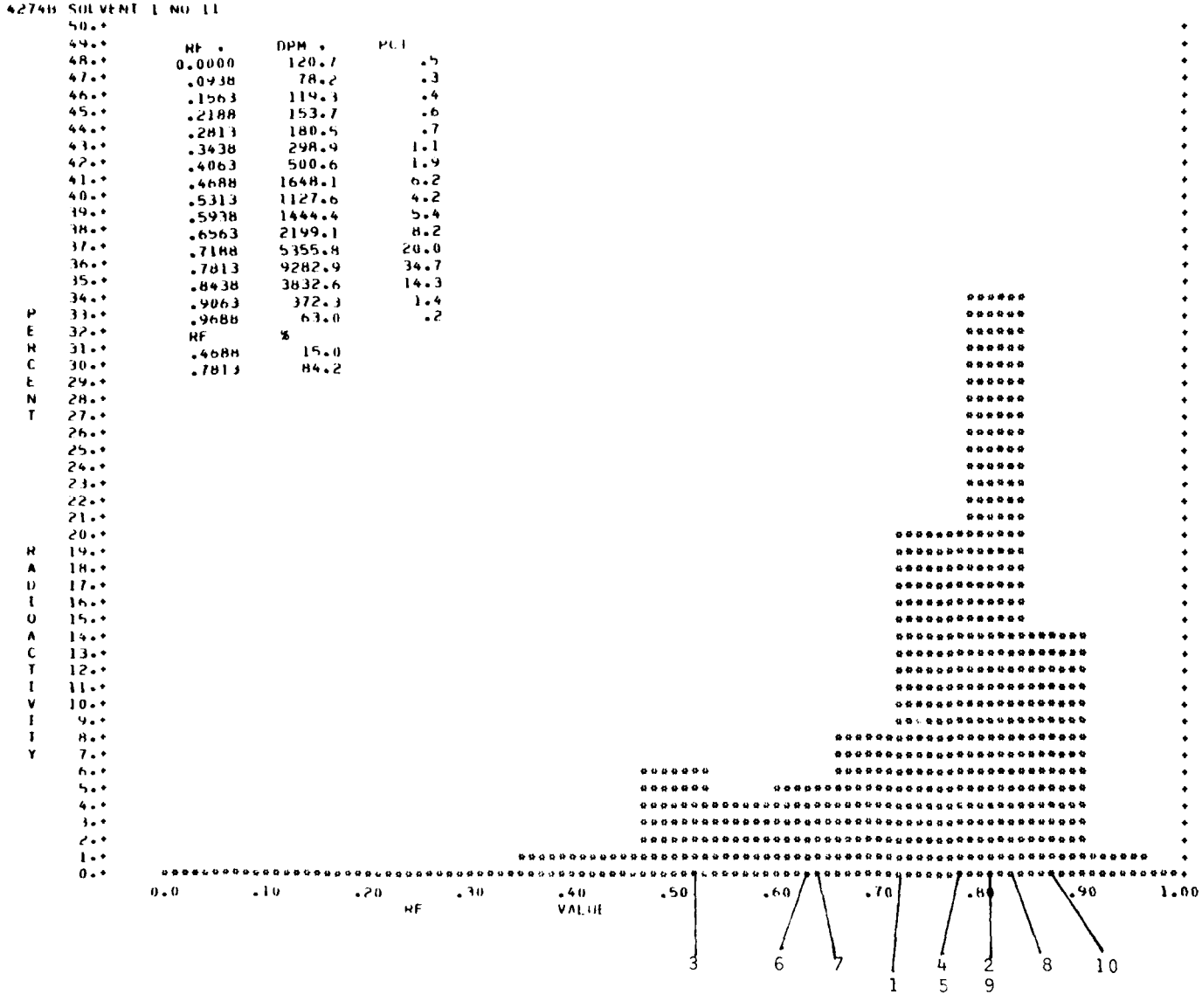


Figure 22-a-I: Oral Treatment, Incubation with Water, Solvent I.

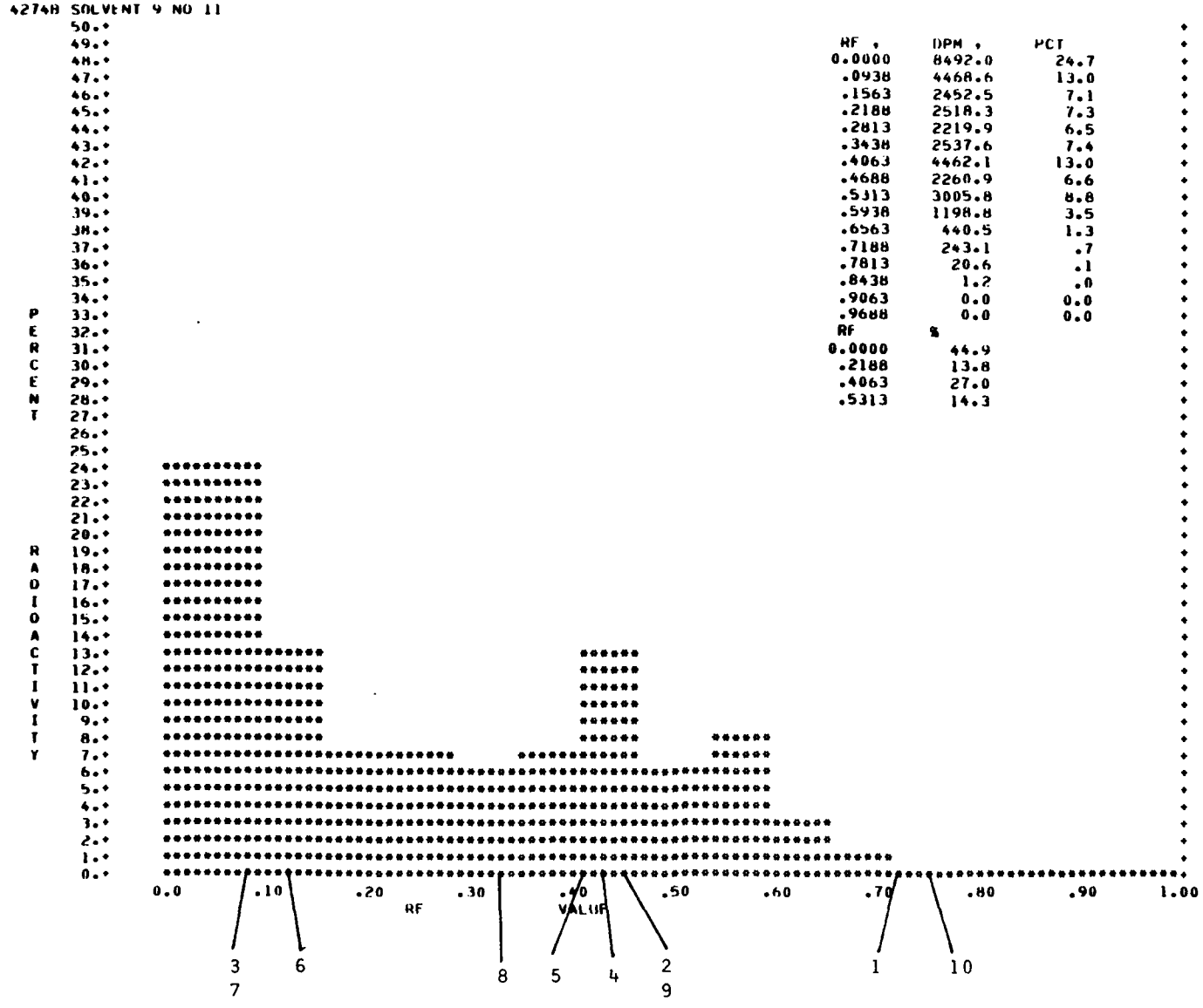


Figure 22-a-IX: Oral Treatment, Incubation with Water, Solvent IX.

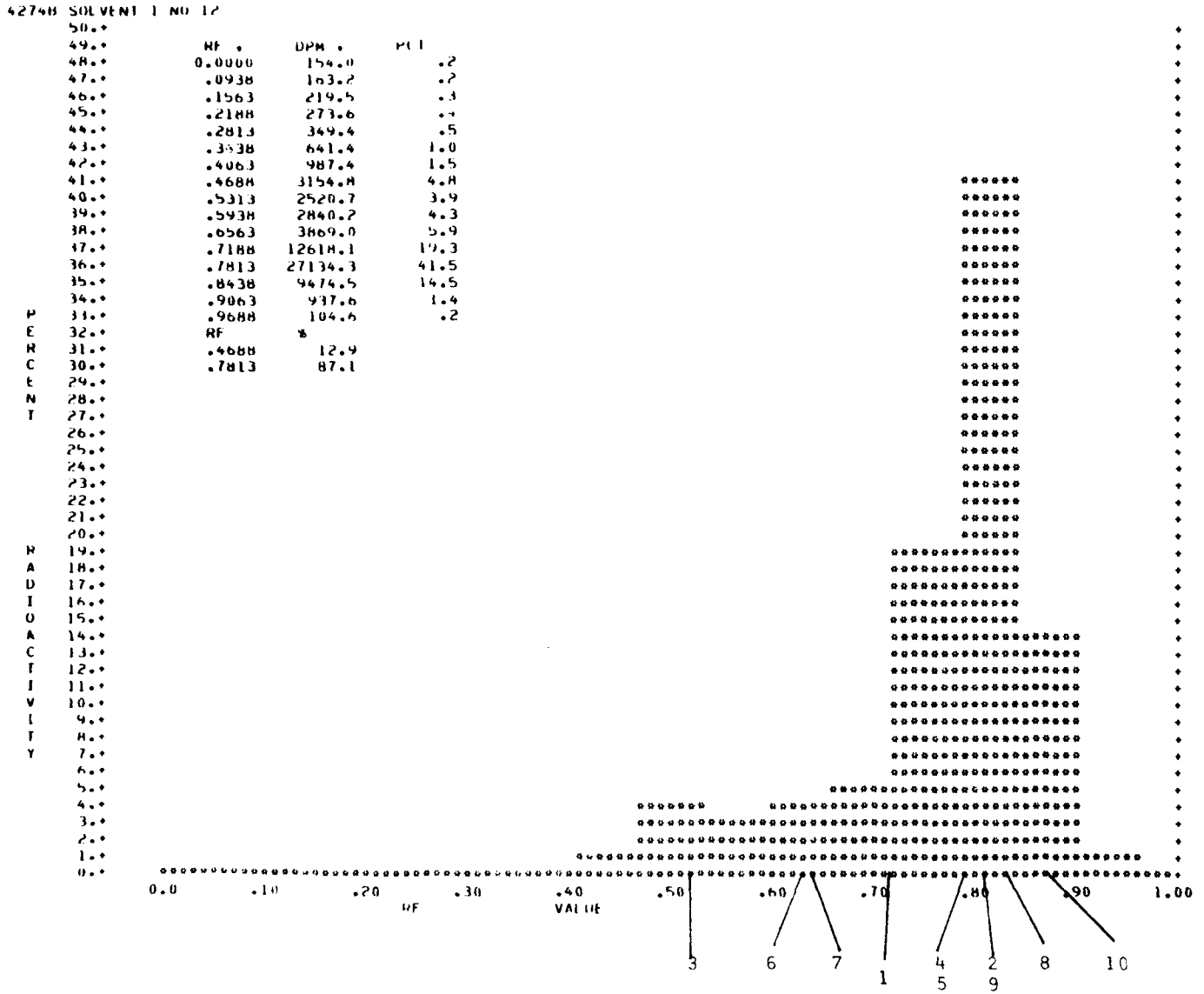


Figure 22-b-I: Oral Treatment, Incubation with B-glucuronidase, Solvent 1.

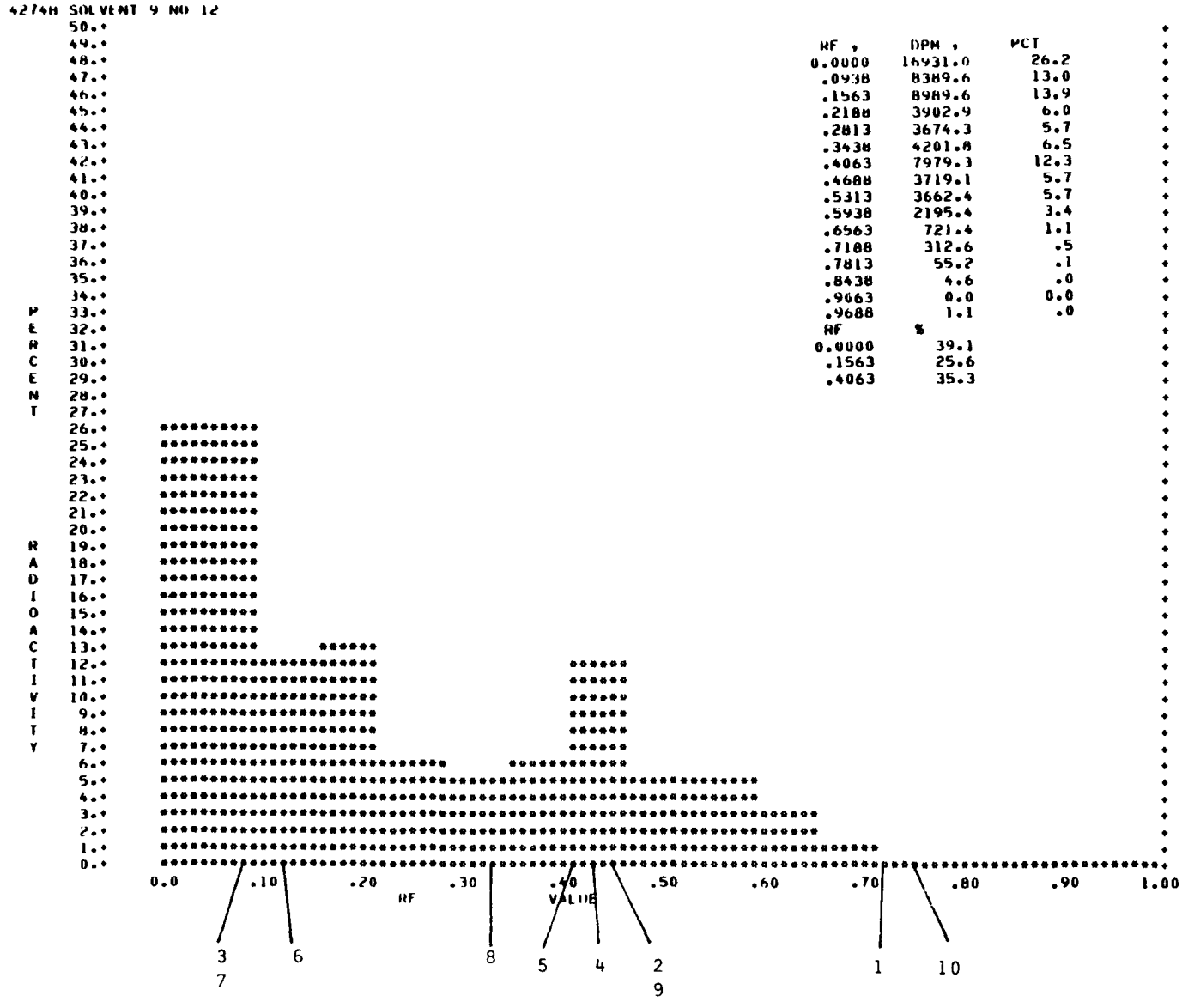


Figure 22-b-IX: Oral Treatment, Incubation with B-glucuronidase, Solvent IX.

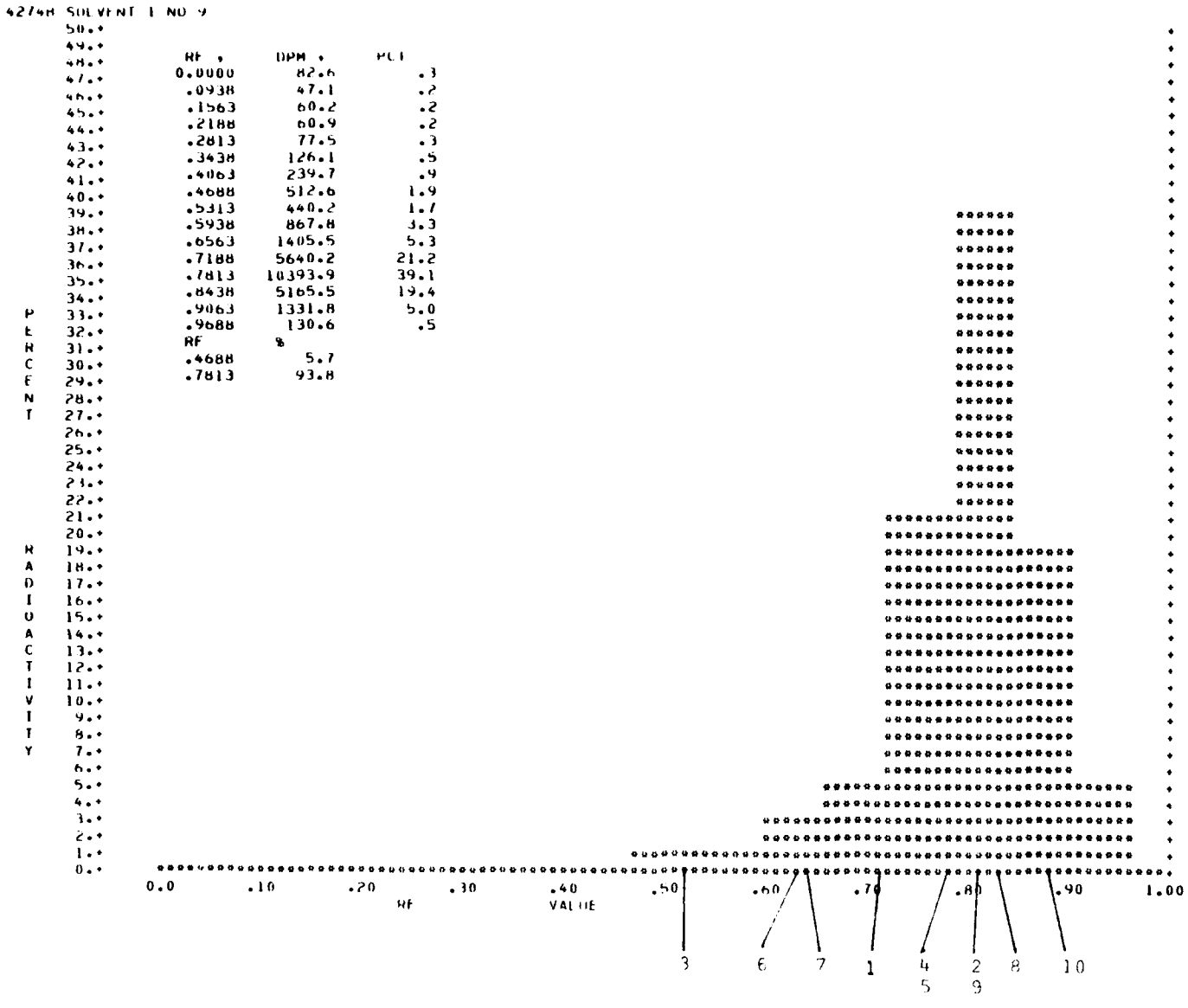


Figure 22-c-I: Dermal Application, Incubation with Water, Solvent I.

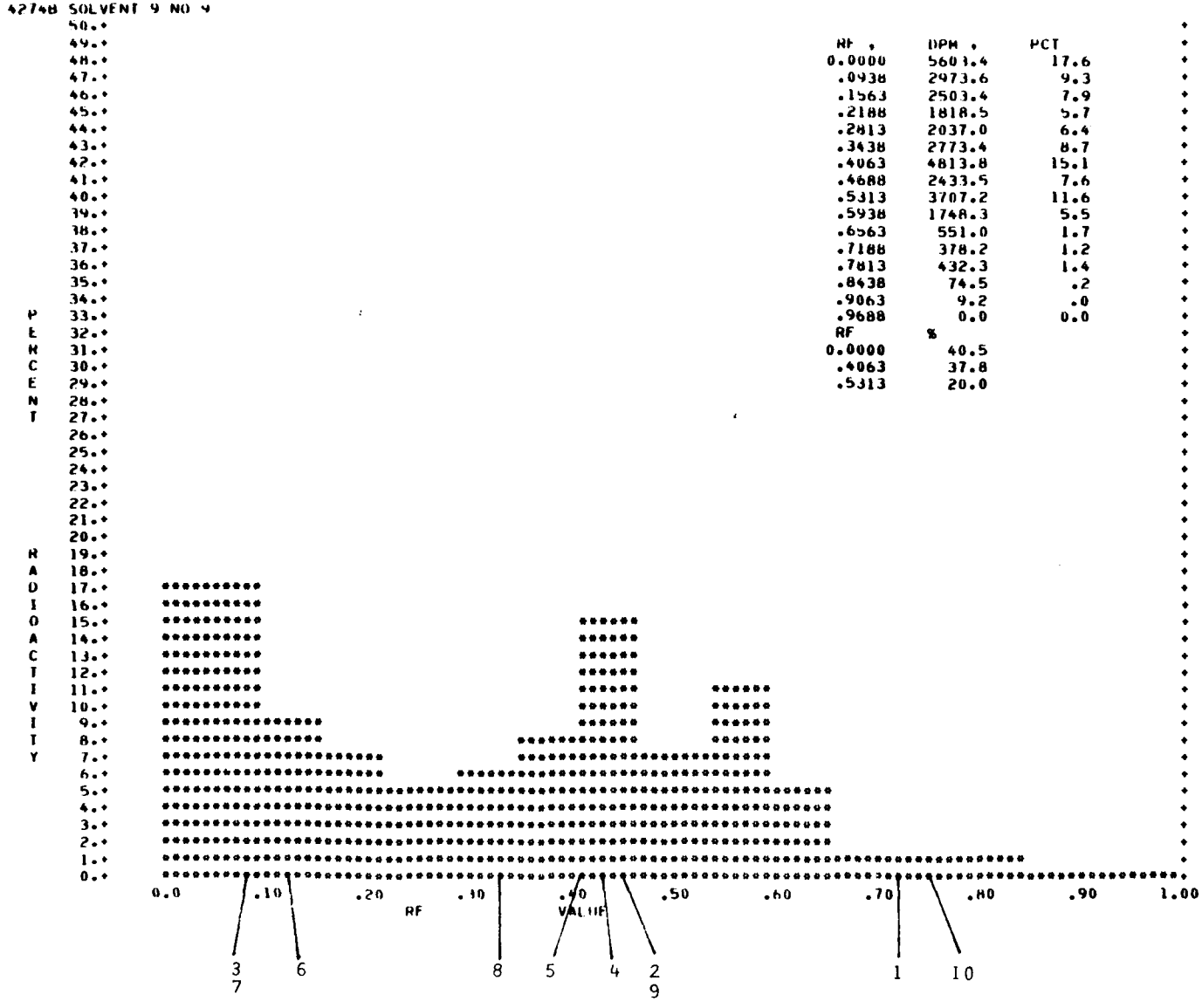


Figure 22-c-IX: Dermal Application, Incubation with Water, Solvent IX.

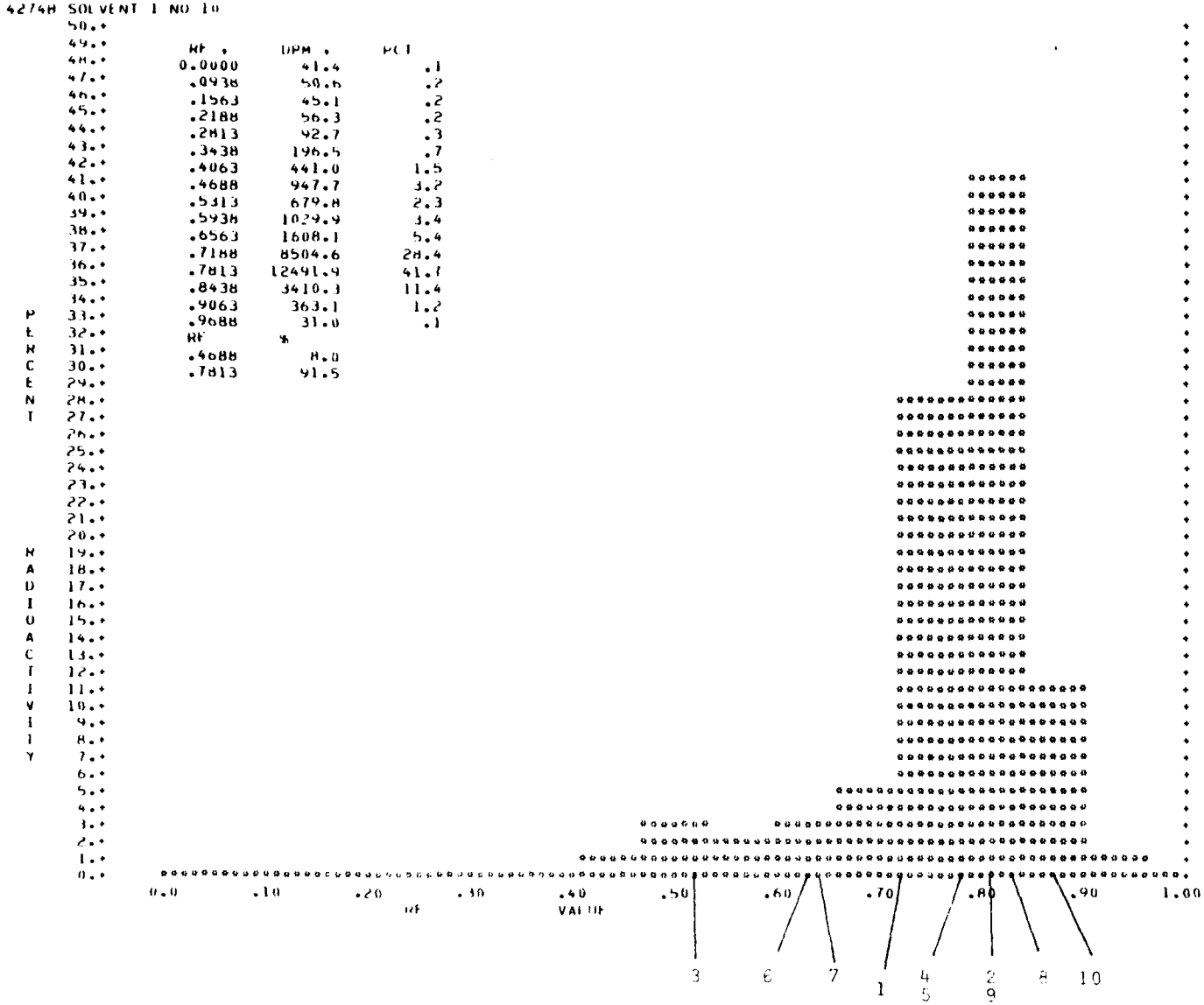


Figure 22-d-1: Dermal Application, Incubation with B-glucuronidase, Solvent 1.

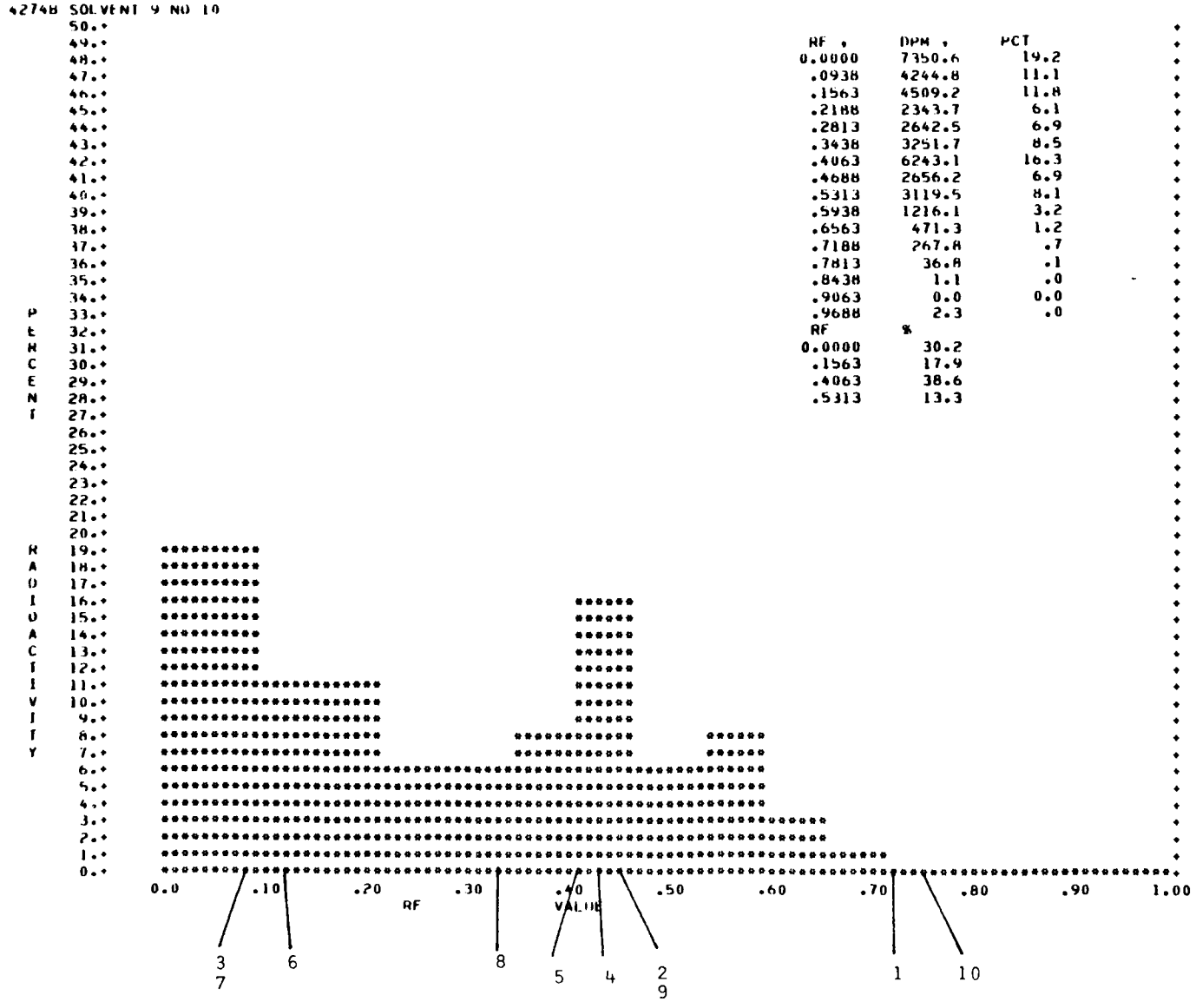


Figure 22-d-IX: Dermal Application, Incubation with B-glucuronidase, Solvent IX.

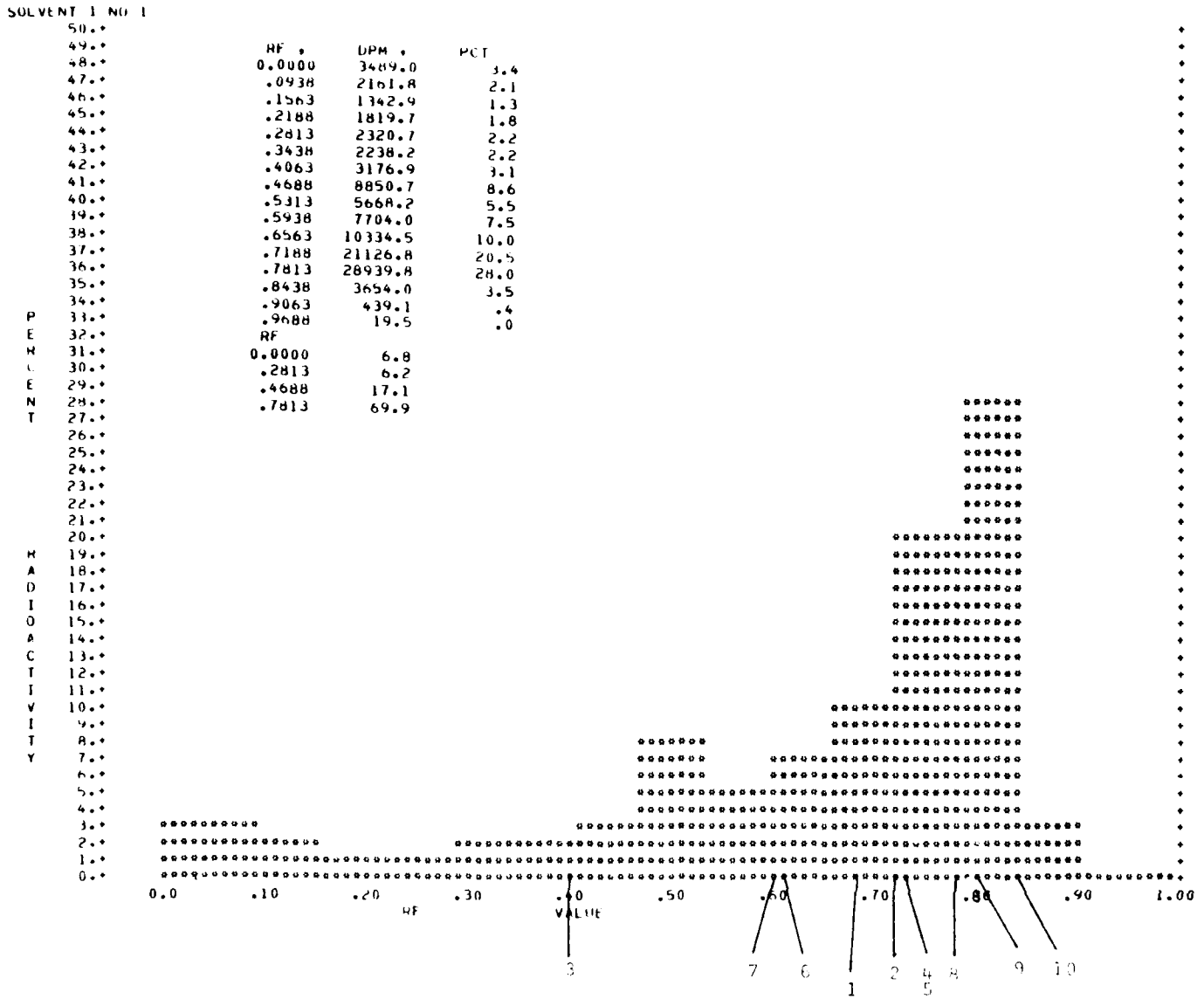


Figure 22-c-1: Oral Treatment, Incubation with Water, Solvent 1.

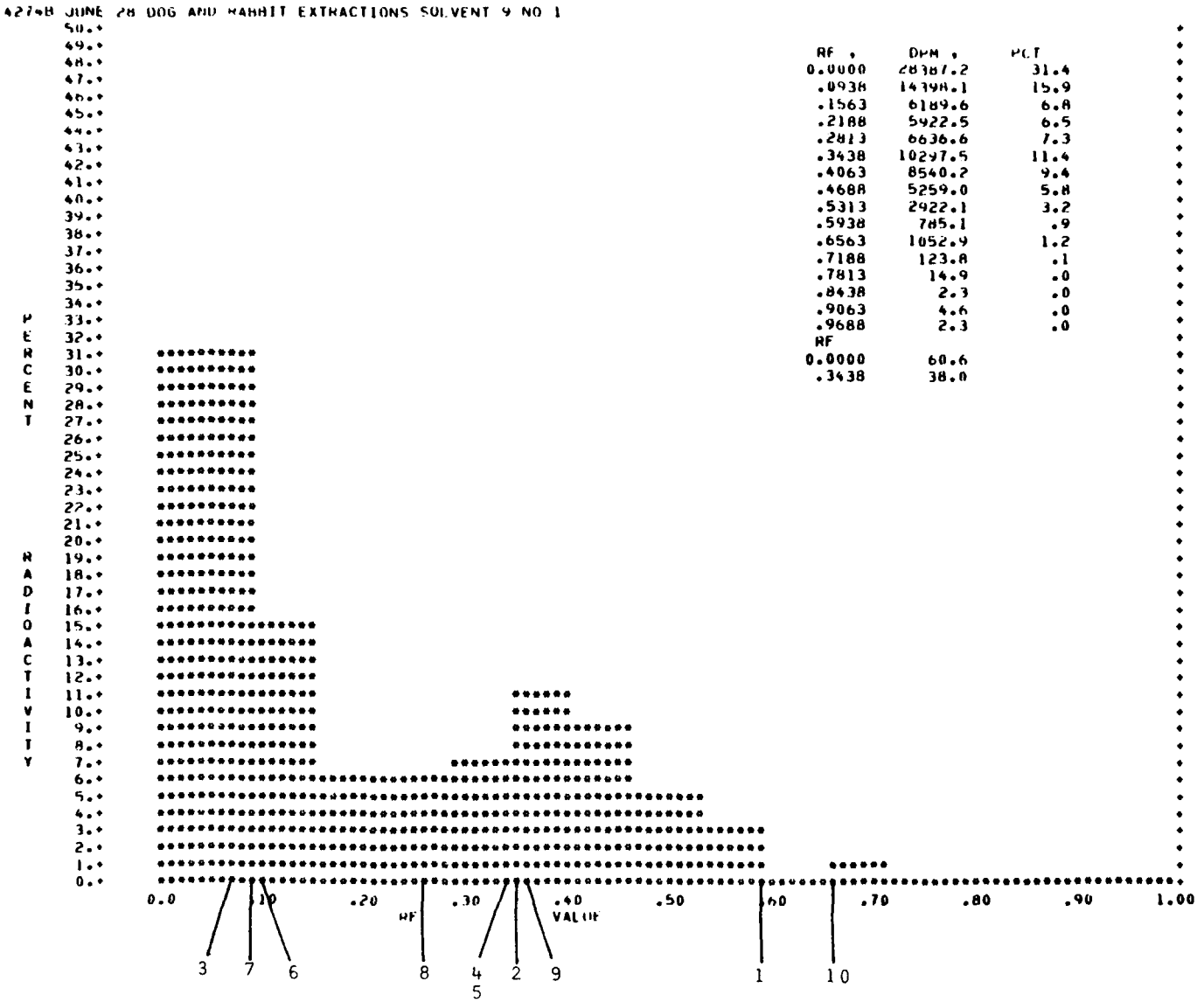


Figure 22-e-IX: Oral Treatment, Incubation with Water, Solvent IX.

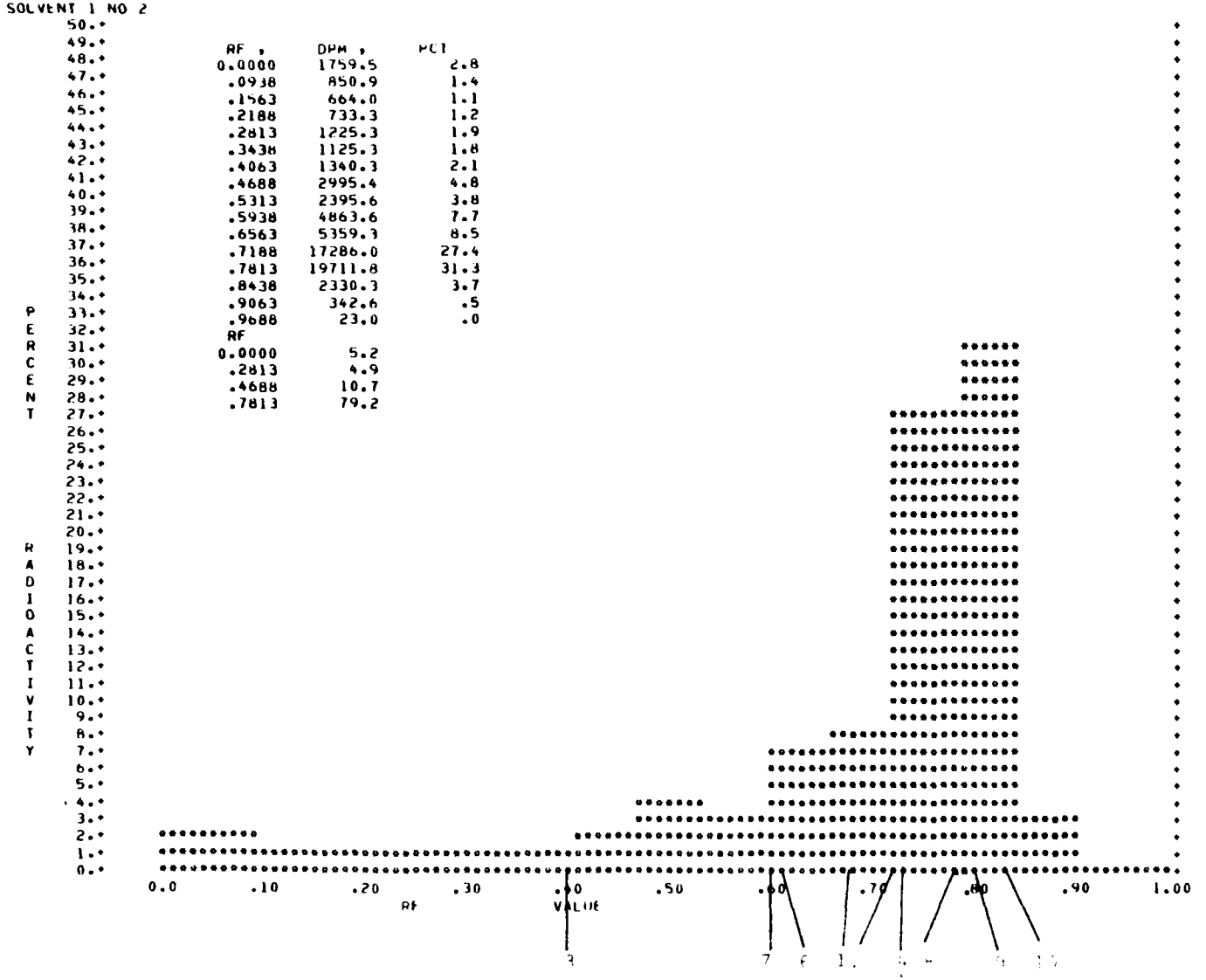


Figure 27-1-1: Oral Treatment, Incubation with B-glucuronidase, Solvent 1.

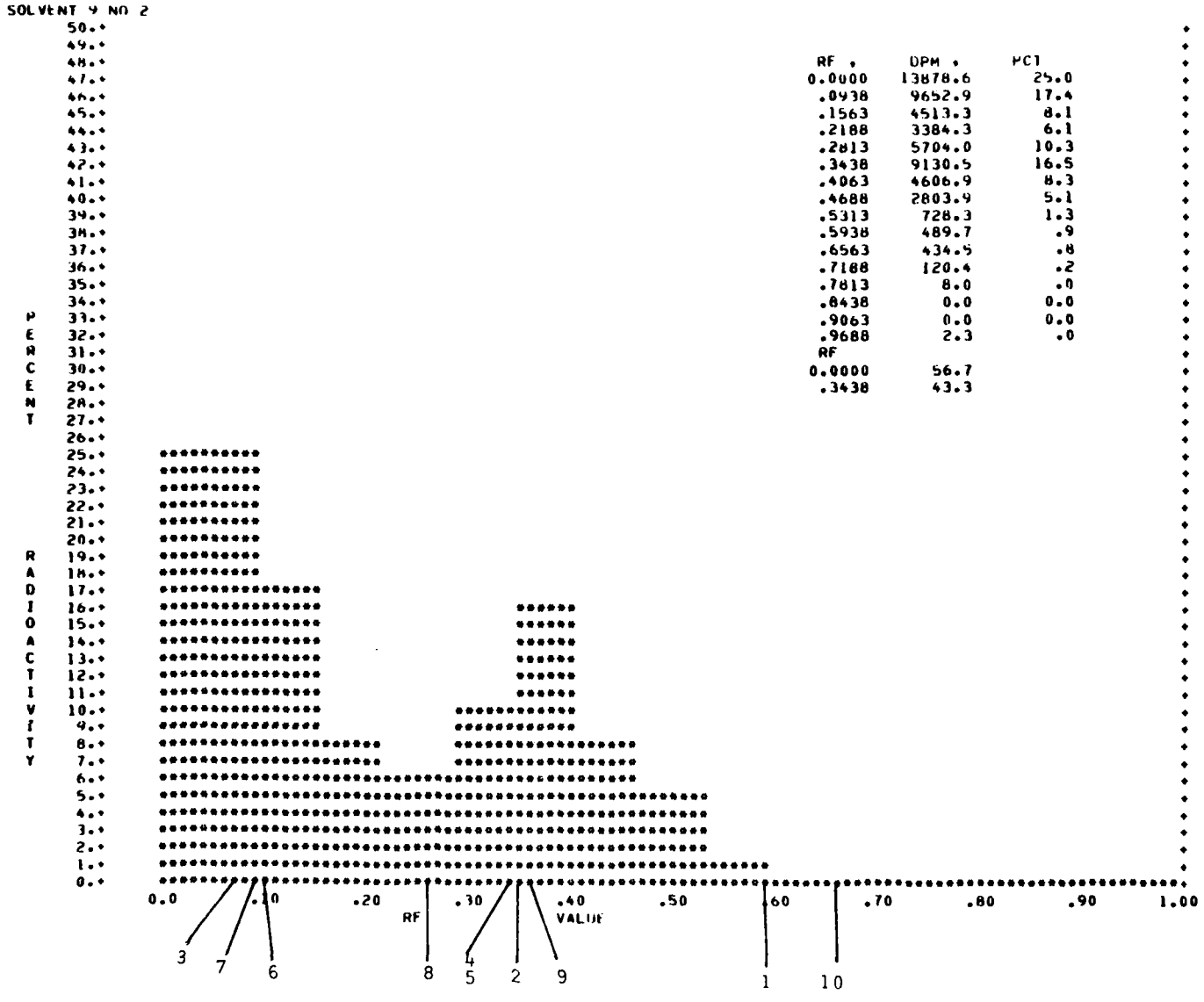


Figure 22-f-IX: Oral Treatment, Incubation with B-glucuronidase, Solvent IX.

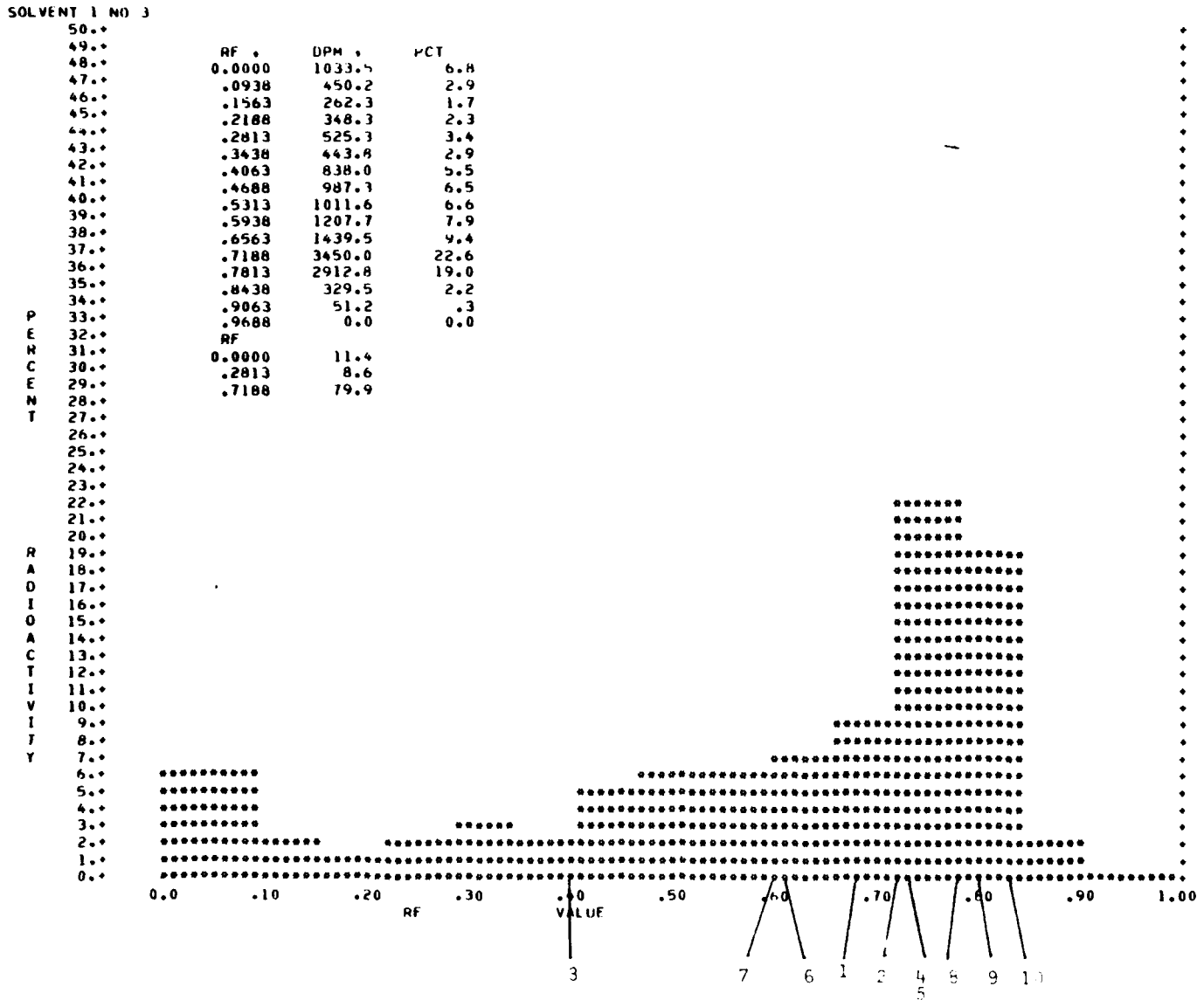


Figure 22-g-1: Dermal Application, Incubation with Water, Solvent 1.

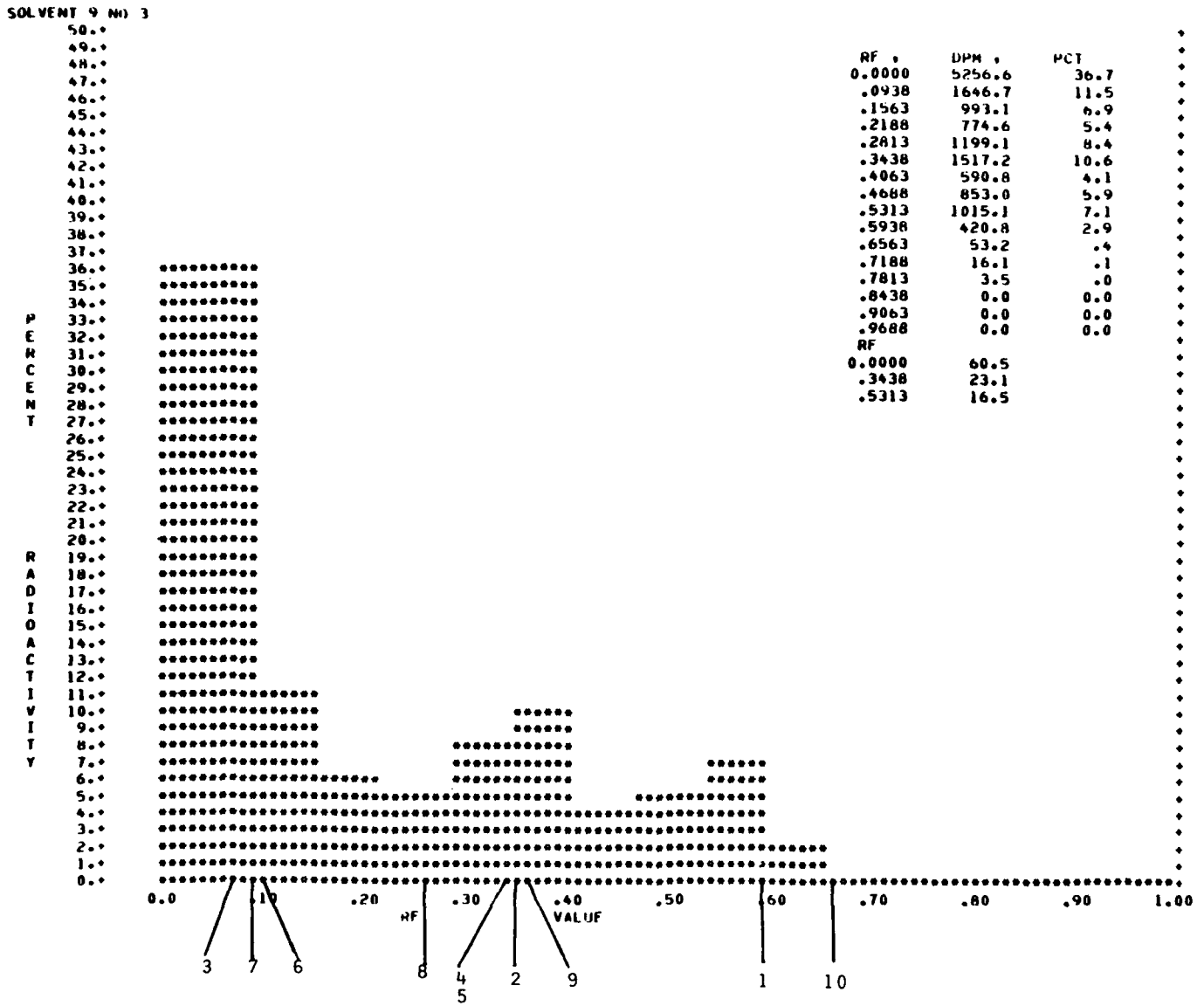


Figure 22-g-IX: Dermal Application, Incubation with Water, Solvent IX.

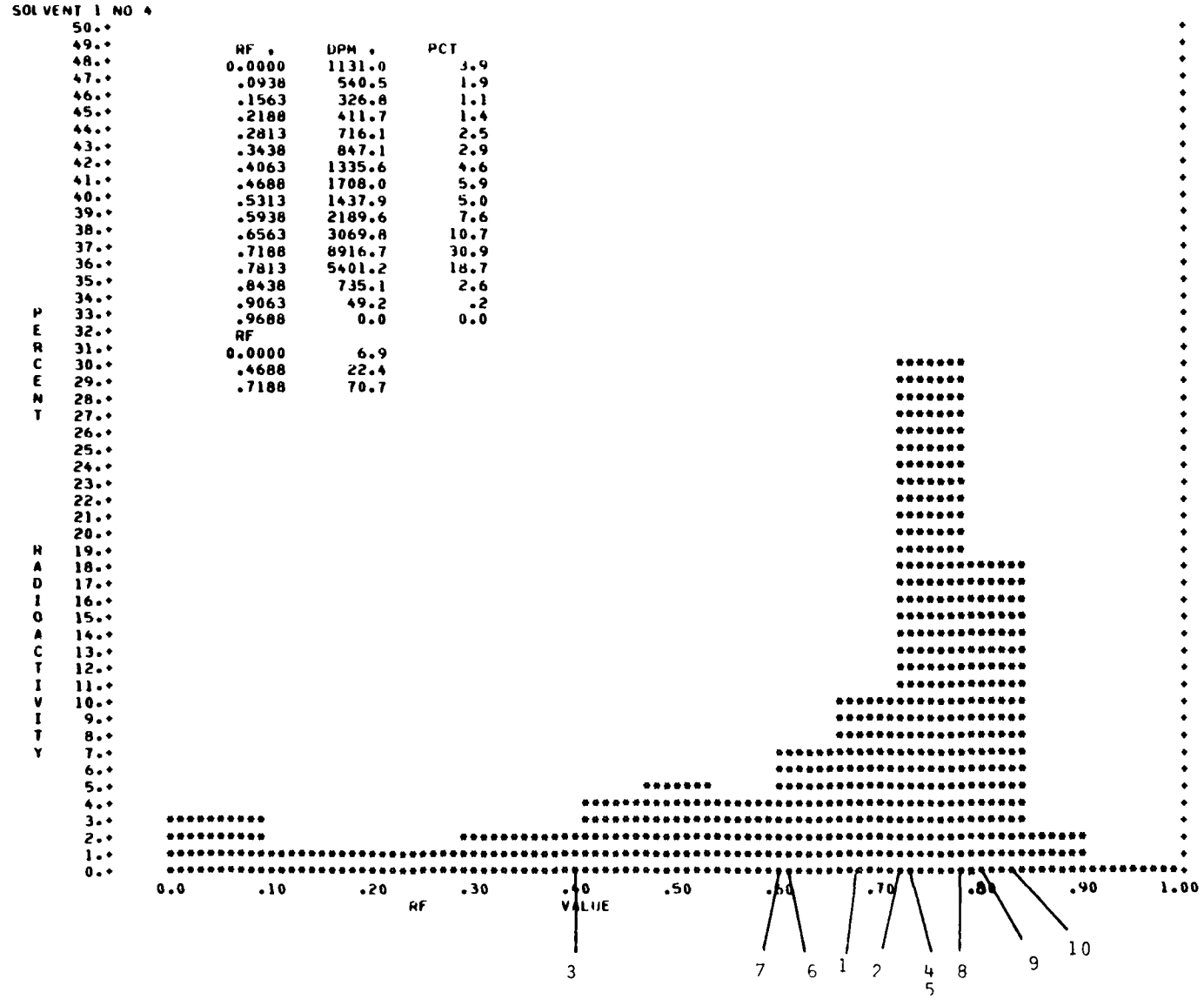


Figure 22-h-I: Dermal Application, Incubation with B-glucuronidase, Solvent I.

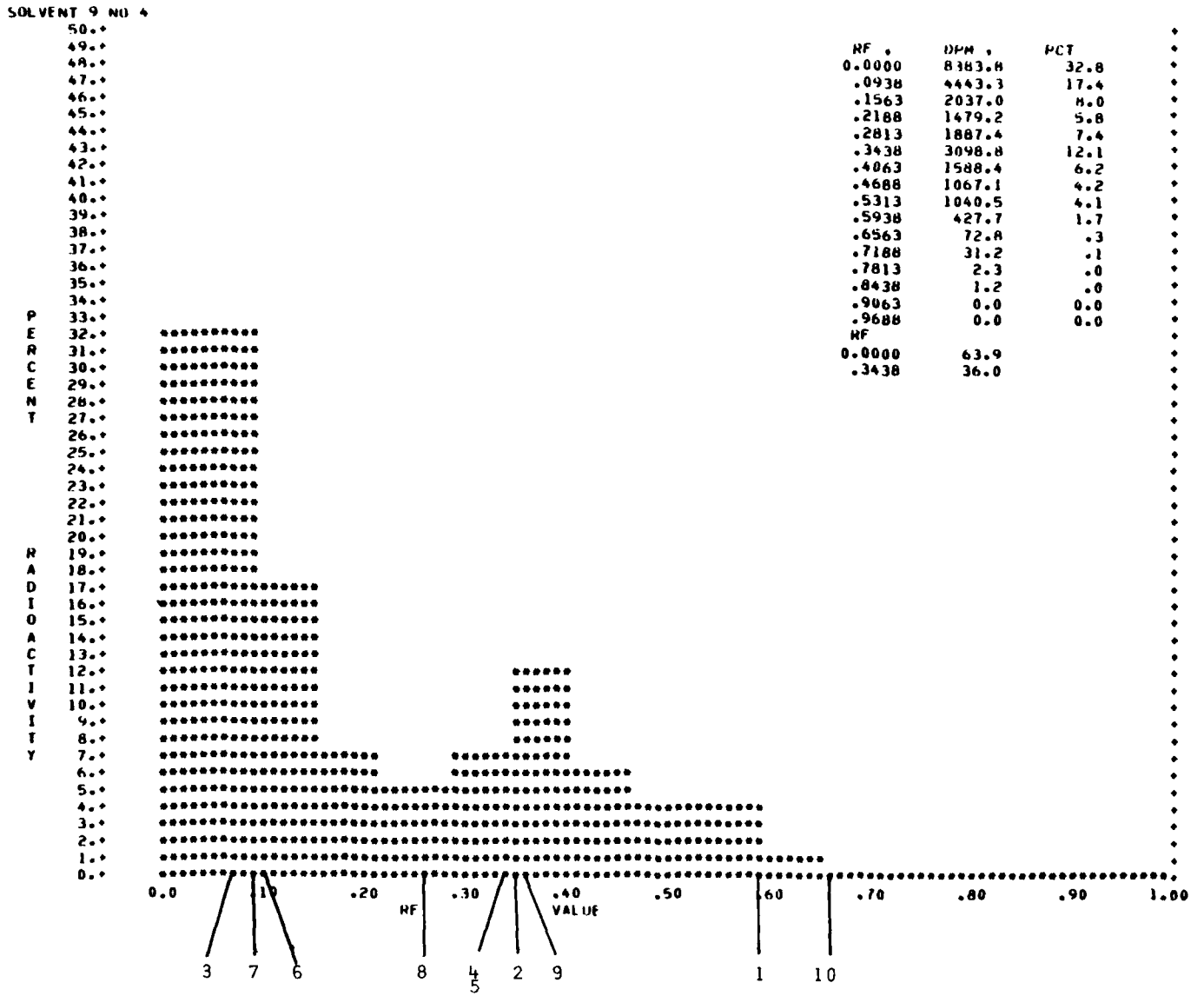


Figure 22-h-IX: Dermal Application, Incubation with B-glucuronidase, Solvent IX.

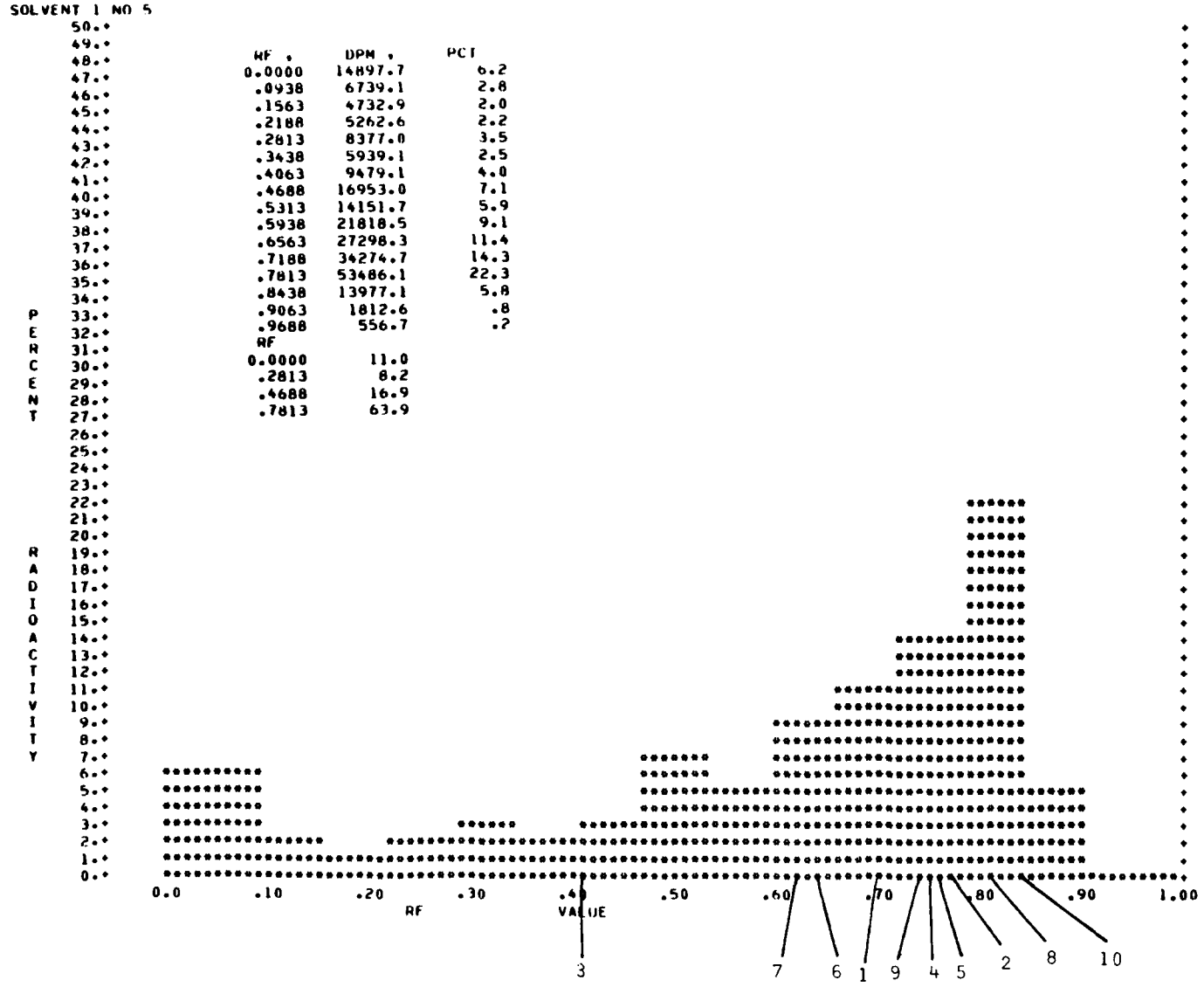


Figure 22-k-I: Oral Treatment, Incubation with Water, Solvent I.

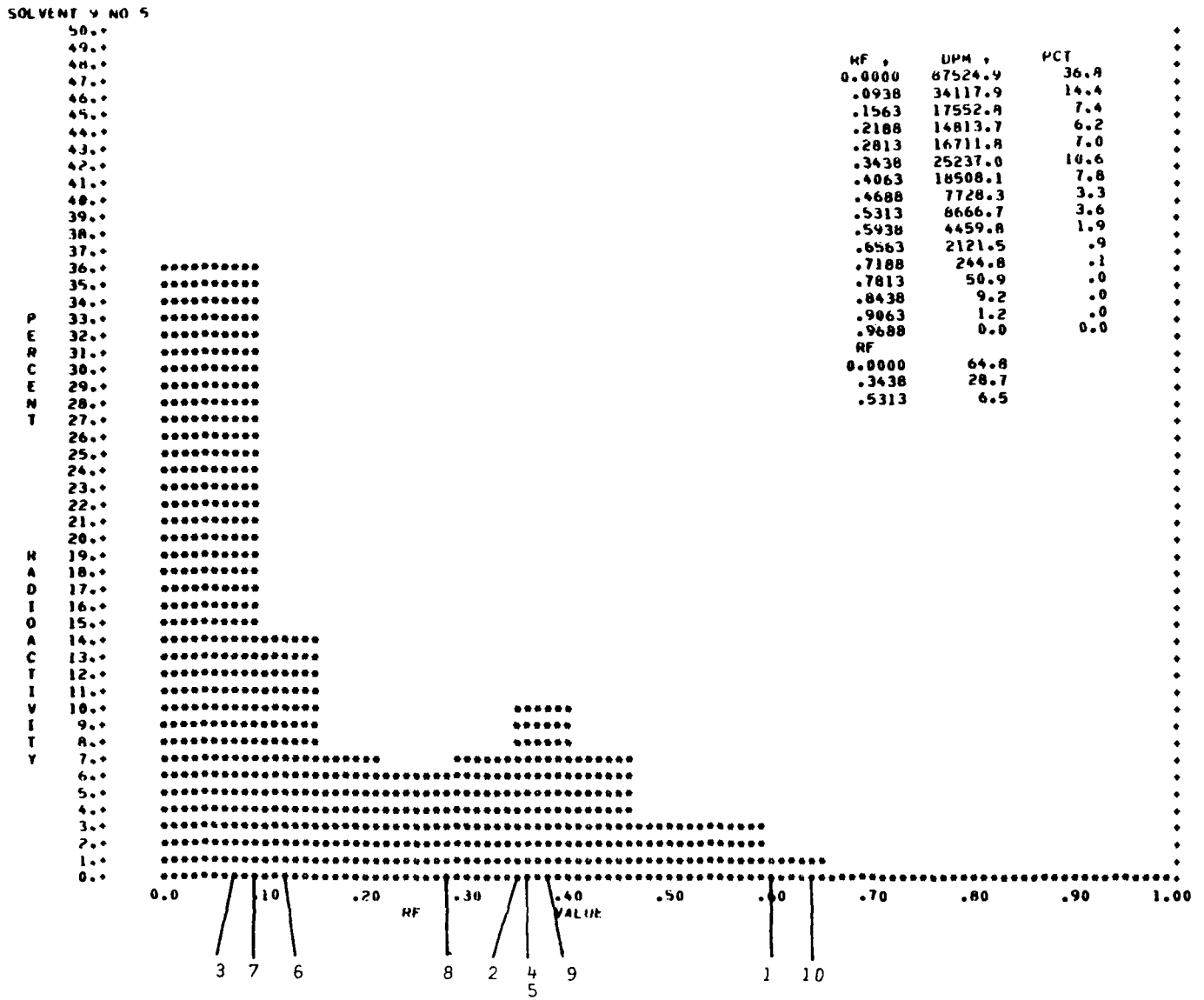
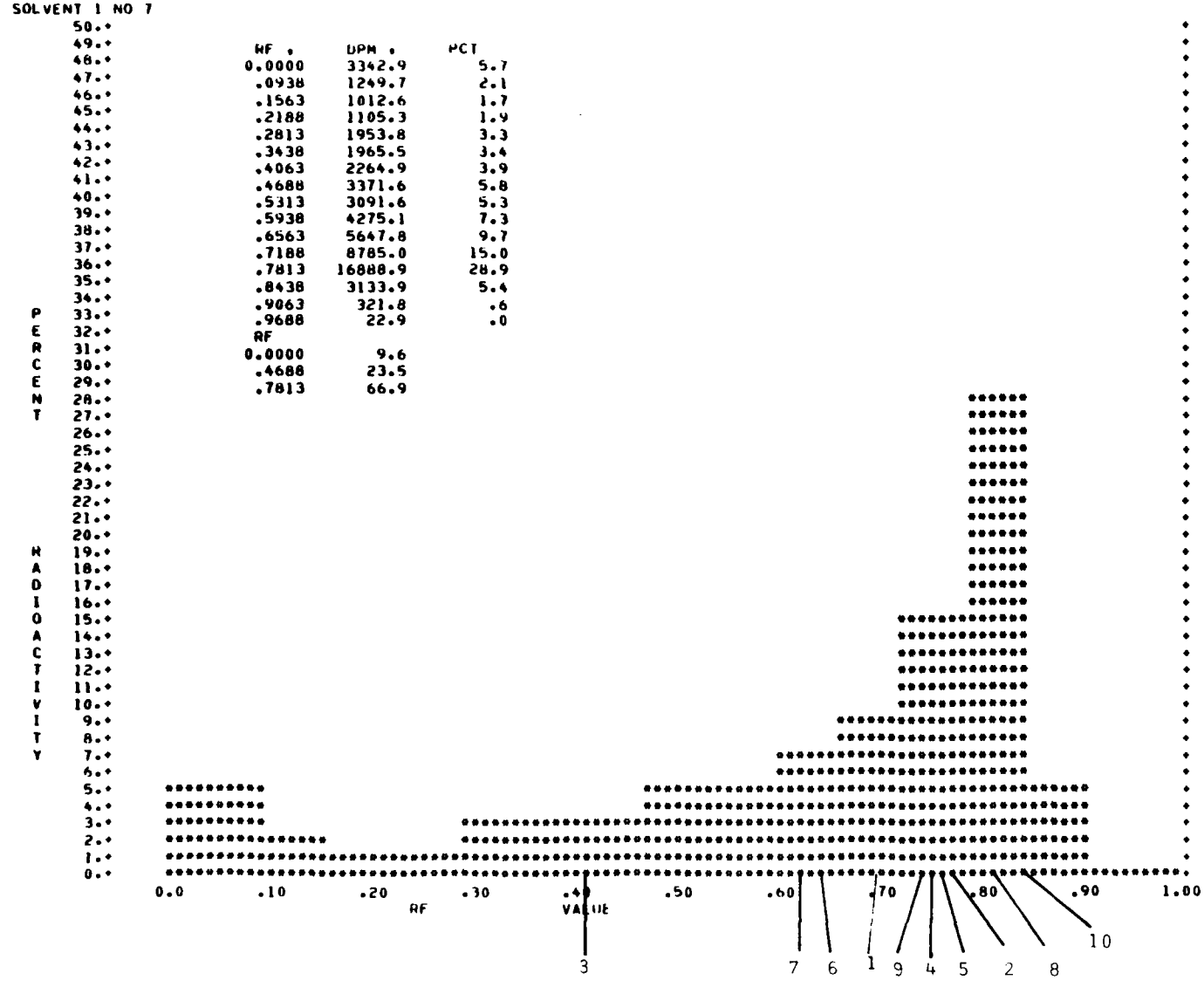


Figure 22-k-IX: Oral Treatment, Incubation with Water, Solvent IX.



RF	DPM	PCT
0.0000	3342.9	5.7
.0938	1249.7	2.1
.1563	1012.6	1.7
.2188	1105.3	1.9
.2813	1953.8	3.3
.3438	1965.5	3.4
.4063	2264.9	3.9
.4688	3371.6	5.8
.5313	3091.6	5.3
.5938	4275.1	7.3
.6563	5647.8	9.7
.7188	8785.0	15.0
.7813	16888.9	28.9
.8438	3133.9	5.4
.9063	321.8	.6
.9688	22.9	.0

RF	
0.0000	9.6
.4688	23.5
.7813	66.9

Figure 22-1-I: Dermal Application, Incubation with Water, Solvent I.

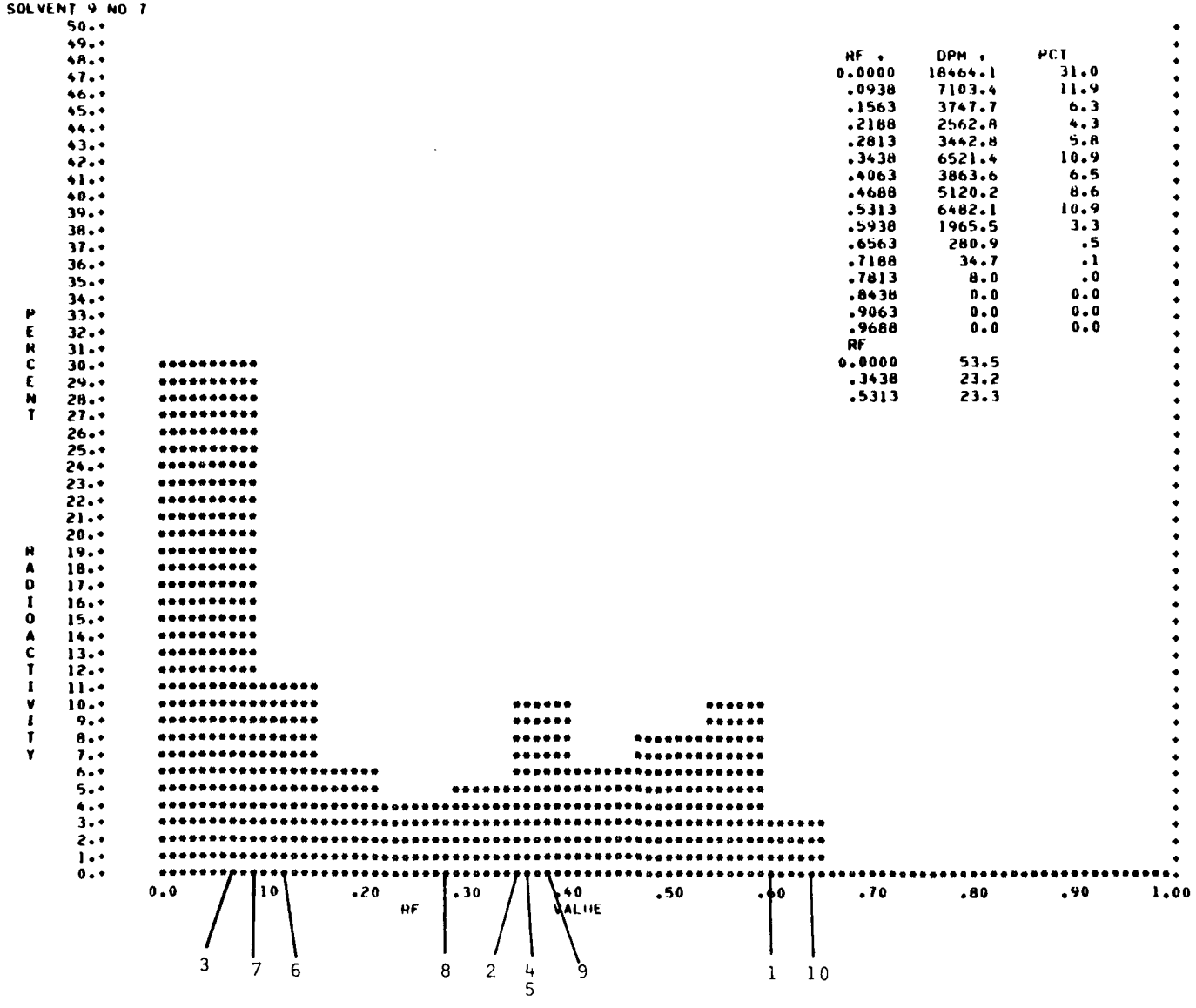


Figure 22-1-IX: Dermal Application, Incubation with Water, Solvent IX.

Figure 23: TLC of the Aqueous Non-Extractable Material Remaining After Extraction of TNT-Urine from Rats, Rabbits and Dogs with Ethyl Acetate. Reference standards are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzylalcohol
3. Trinitrobenzoic Acid
4. 4-Amino-2,6-Dinitrotoluene
5. 2-Amino-4,6-Dinitroboluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2,6'-Tetranitro-4,4'-azoxytoluene

Figure 23 follows

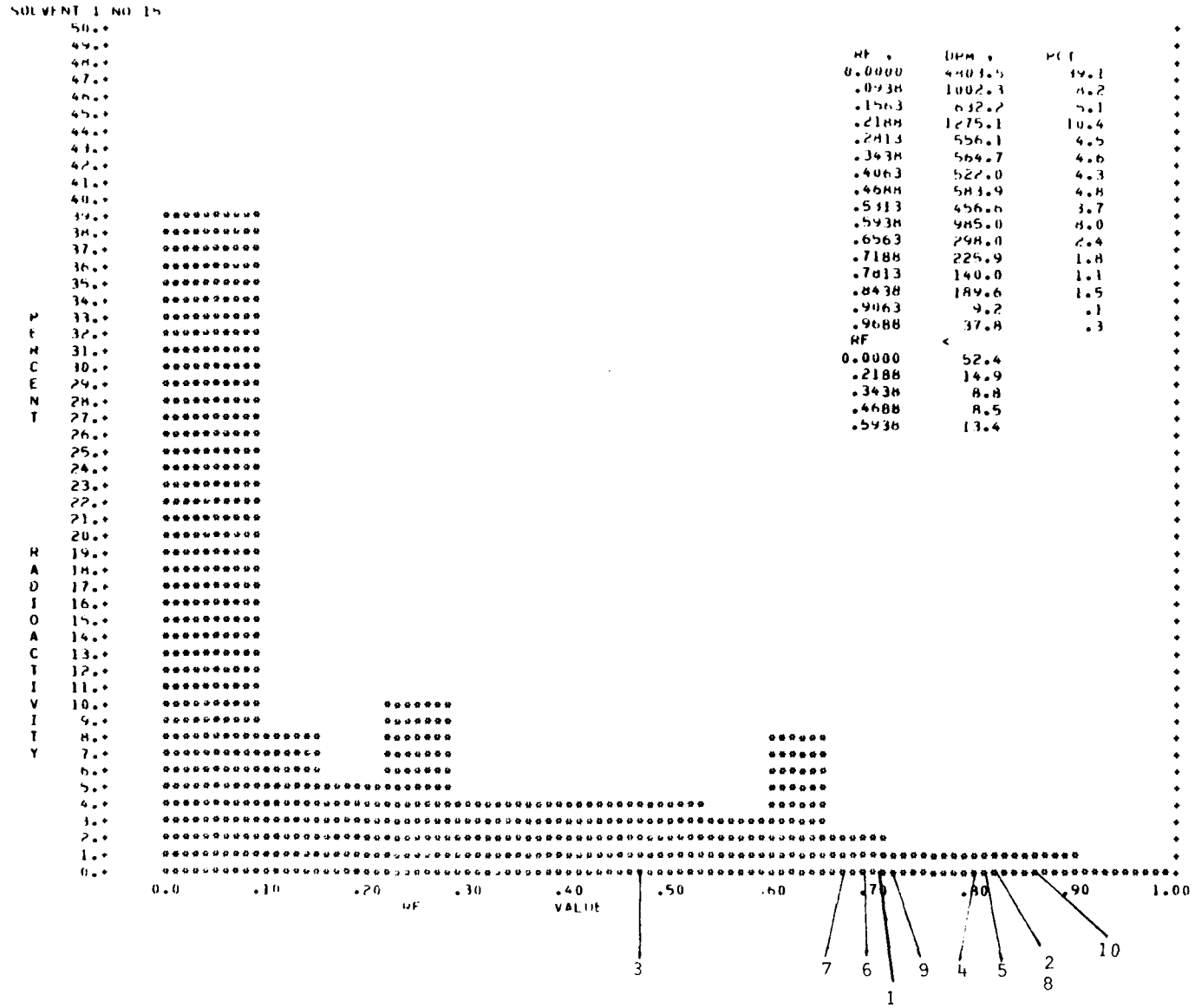


Figure 23-a-I: Male Rats, Oral Treatment, Solvent I

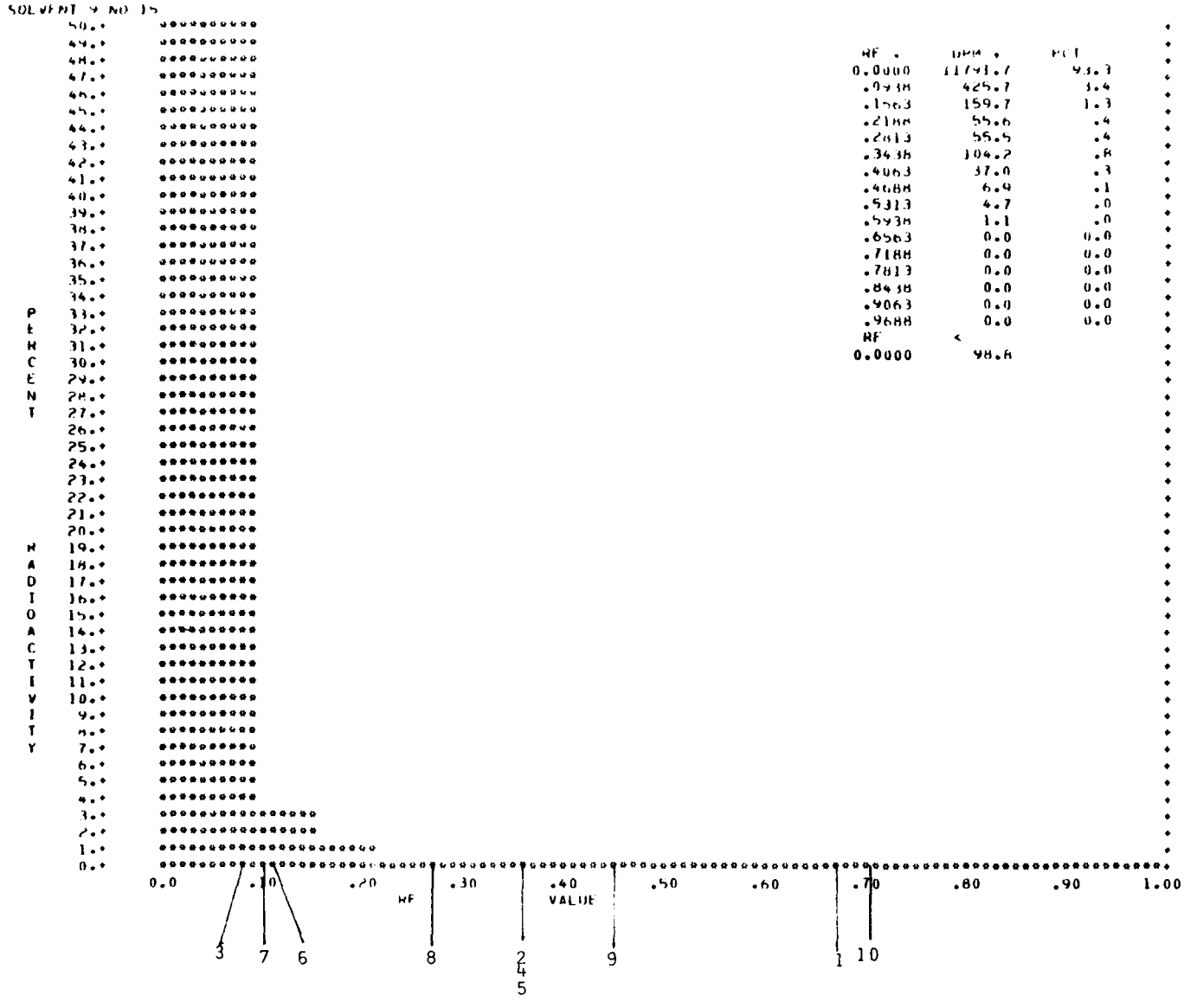


Figure 23-a-IX: Male Rats, Oral Treatment, Solvent IX

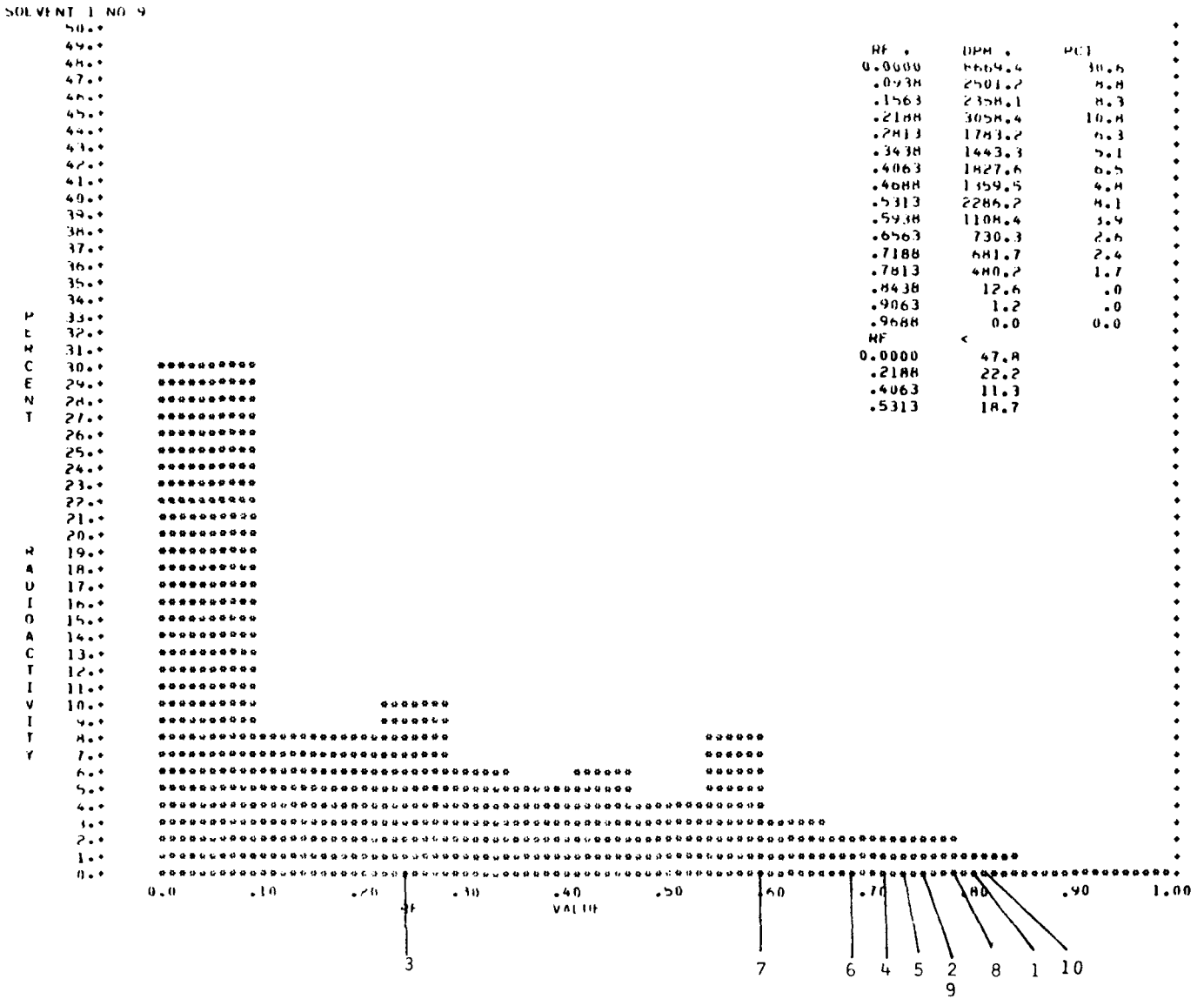


Figure 23-b-I: Female Rats, Oral Treatment, Solvent I

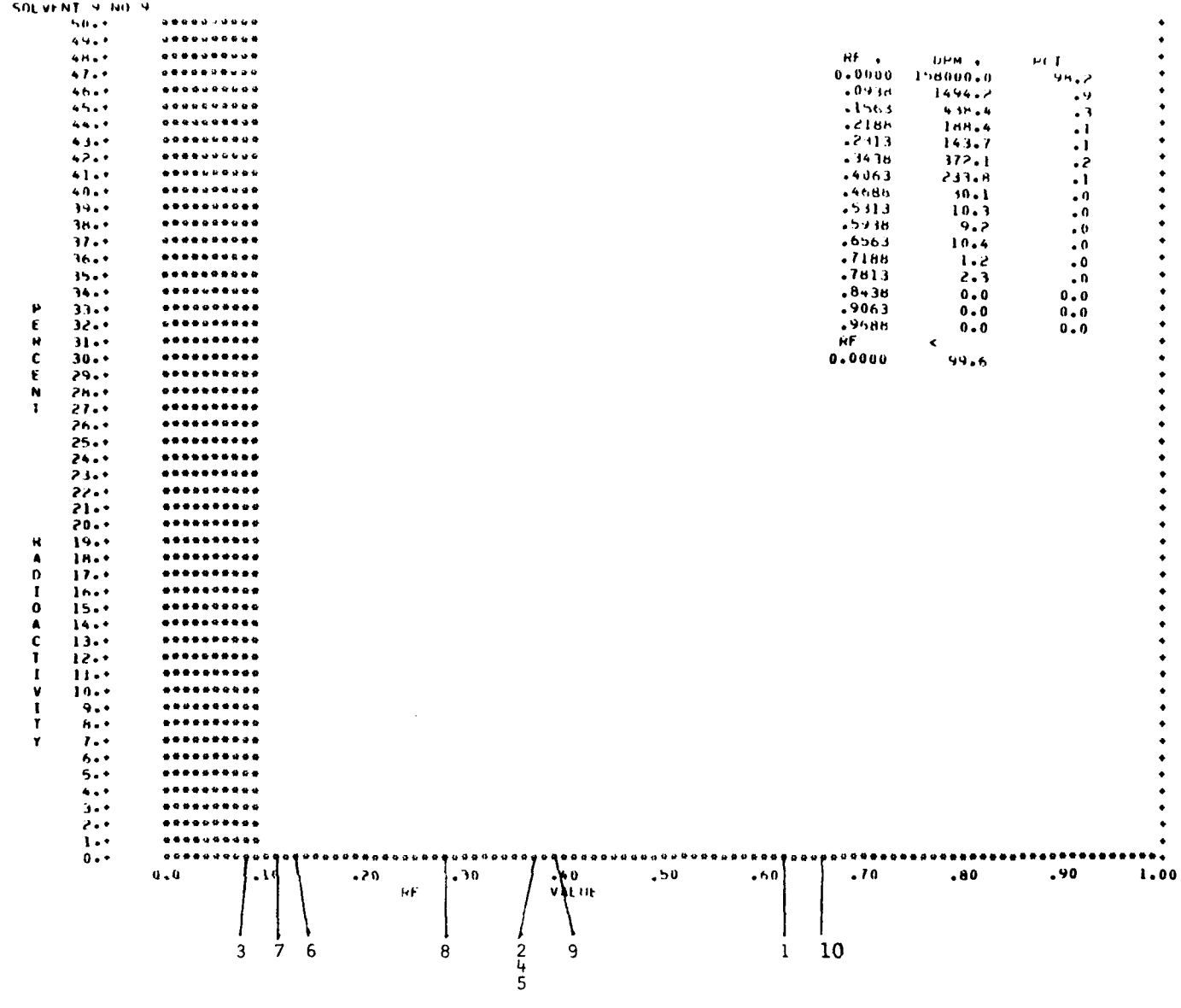
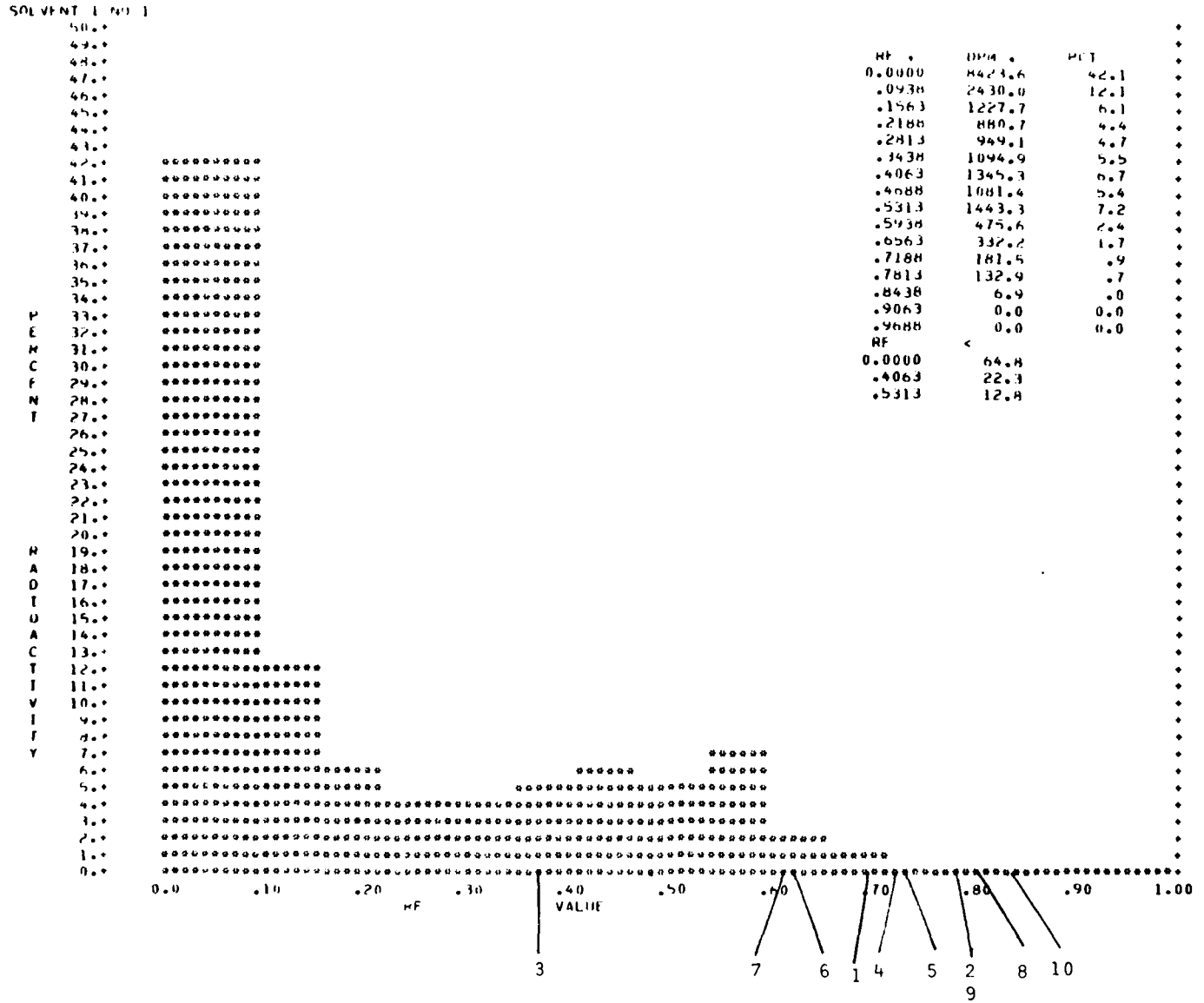


Figure 23-b-IX: Female Rats, Oral Treatment, Solvent IX



RF	DPM	PCT
0.0000	8424.6	42.1
.0938	2430.0	12.1
.1563	1227.7	6.1
.2188	880.7	4.4
.2813	949.1	4.7
.3438	1094.9	5.5
.4063	1345.3	6.7
.4688	1081.4	5.4
.5313	1443.3	7.2
.5938	475.6	2.4
.6563	332.2	1.7
.7188	181.5	.9
.7813	132.9	.7
.8438	6.9	.0
.9063	0.0	0.0
.9688	0.0	0.0

RF	<
0.0000	64.8
.4063	22.3
.5313	12.8

Figure 23-c-I: Male Rats, Dermal Application, Solvent I

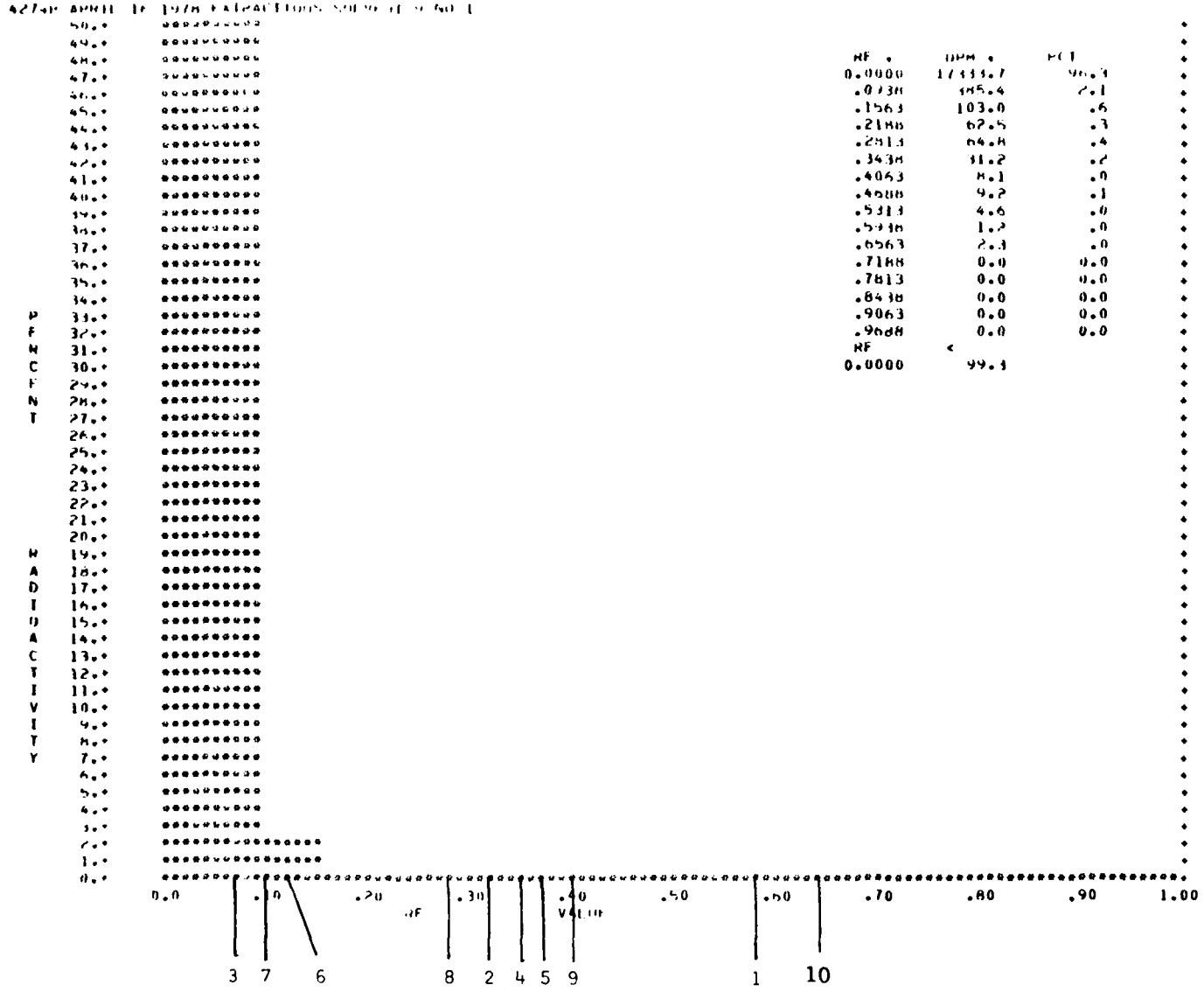


Figure 23-c-IX: Male Rats, Dermal Application, Solvent I

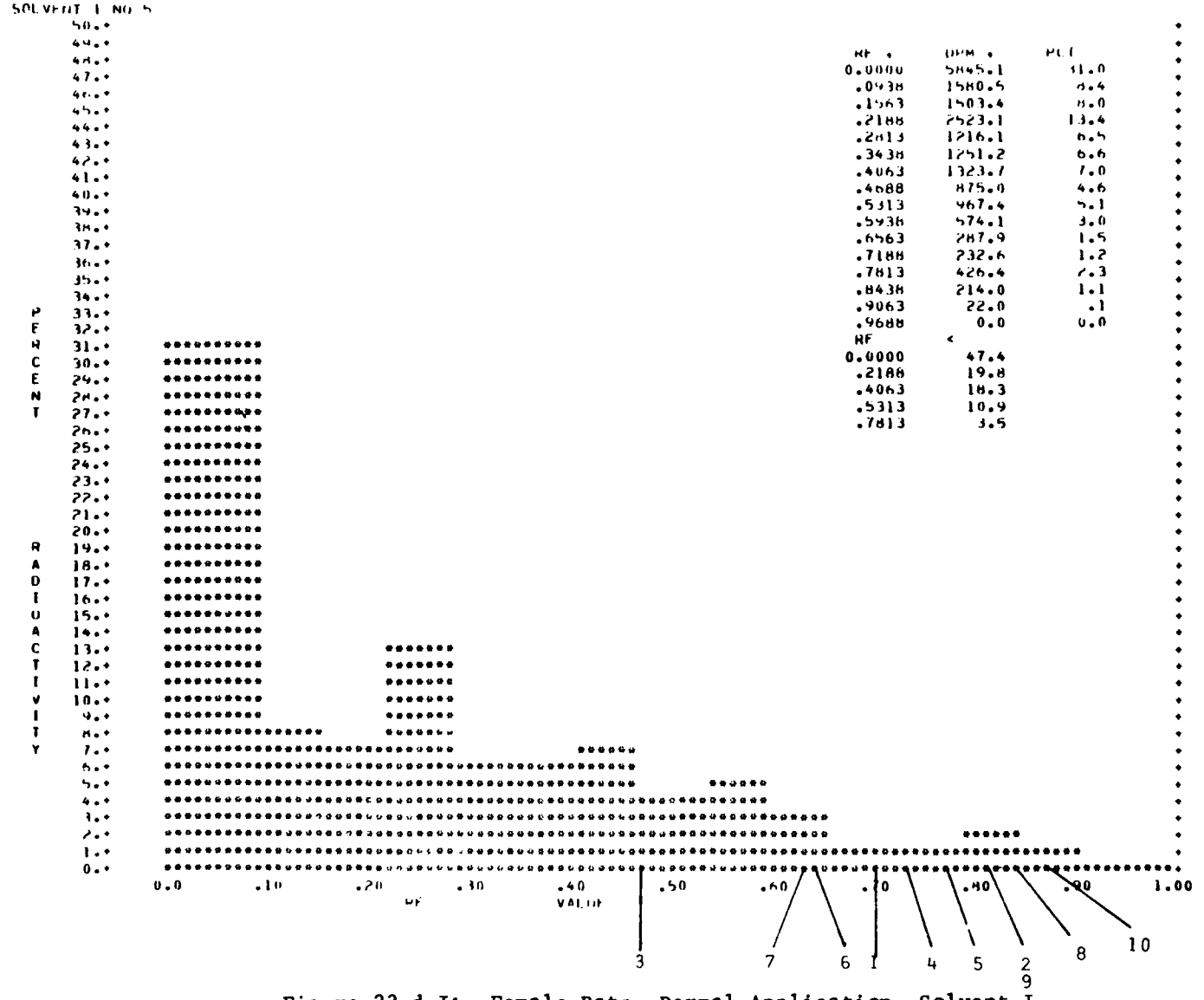


Figure 23-d-I: Female Rats, Dermal Application, Solvent I

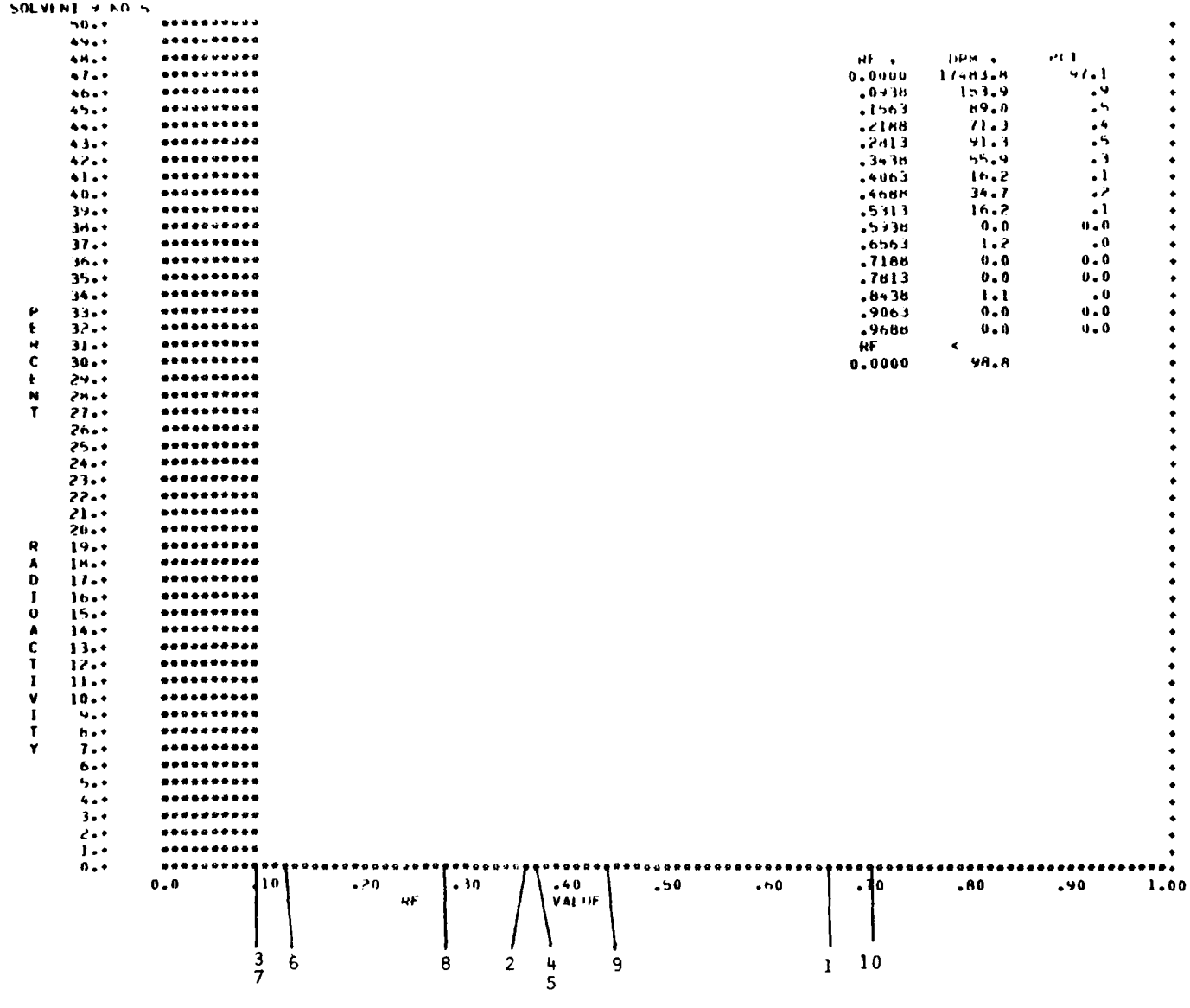


Figure 23-d-IX: Female Rats, Dermal Application, Solvent IX

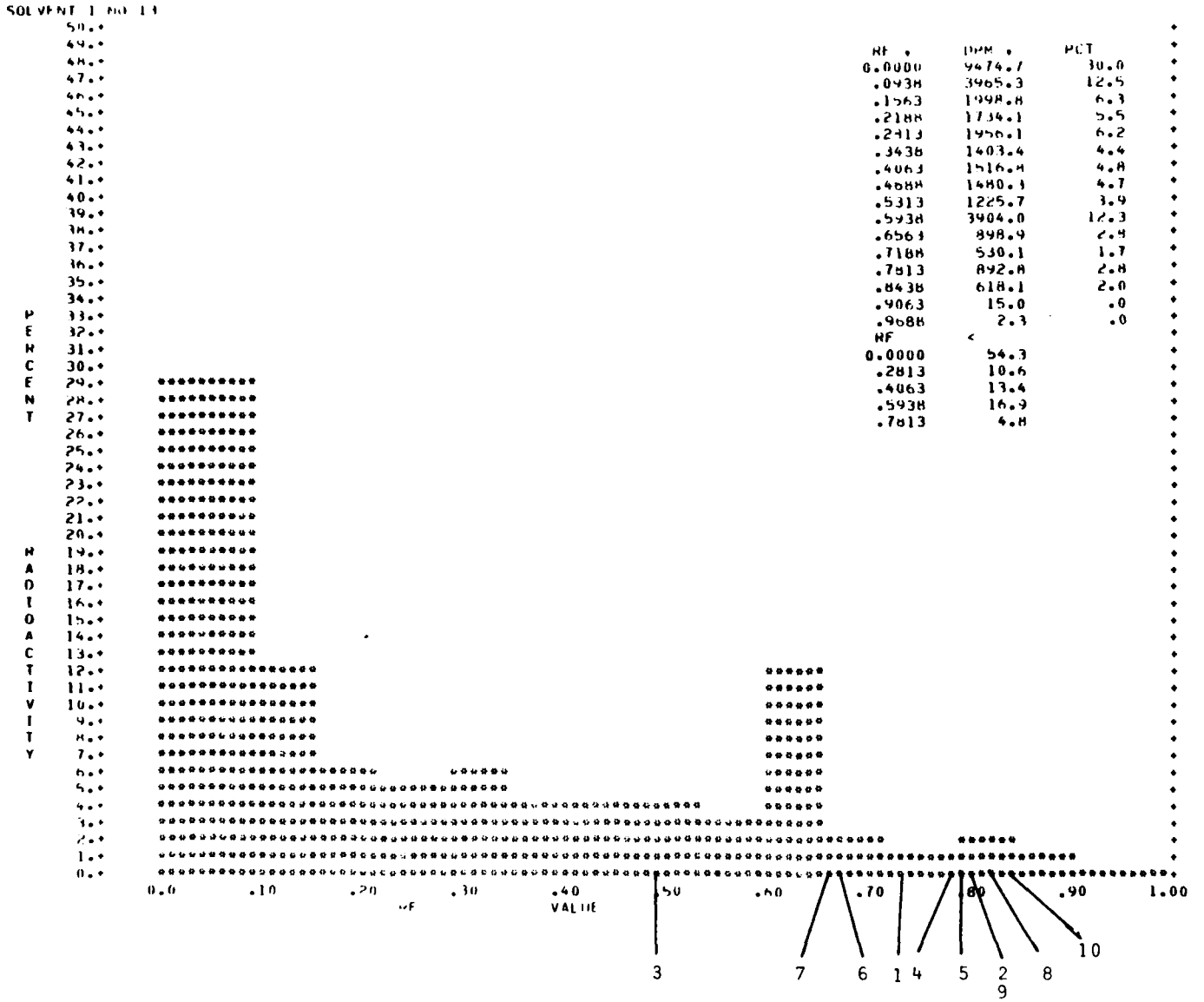


Figure 23-e-I: Male Rabbits, Oral Treatment, Solvent I

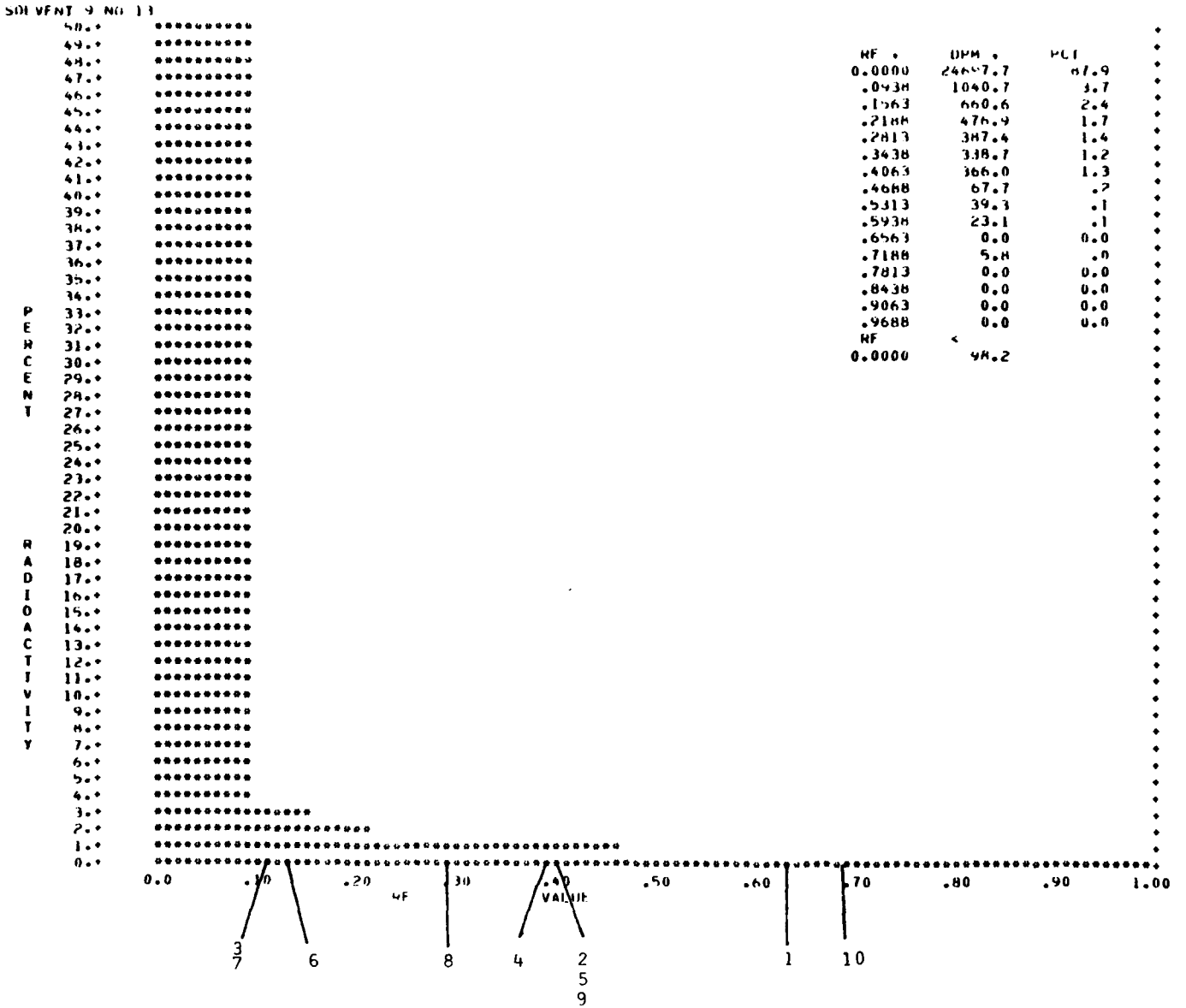


Figure 23-e-IX: Male Rabbits, Oral Treatment, Solvent IX

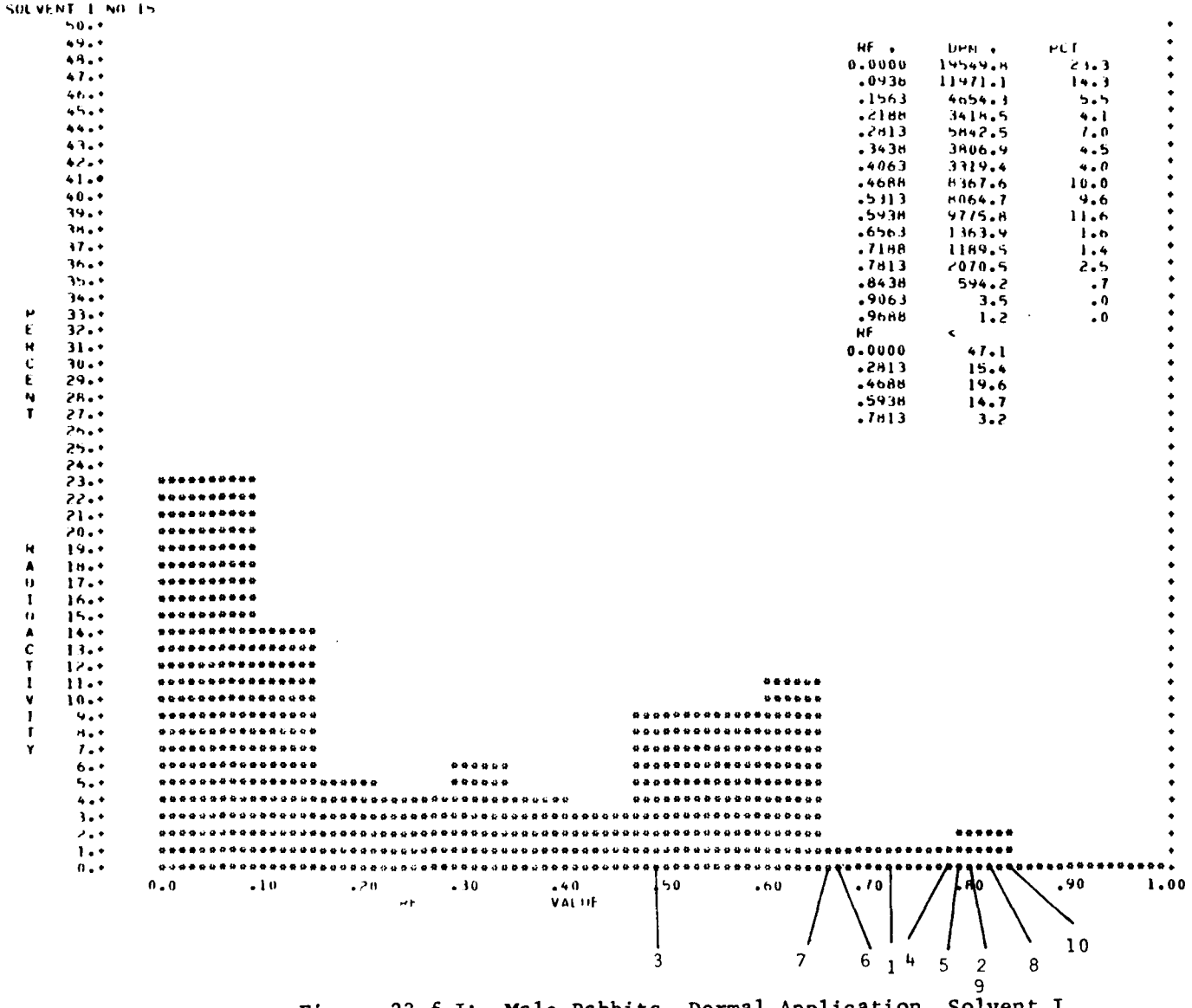


Figure 23-f-I: Male Rabbits, Dermal Application, Solvent I

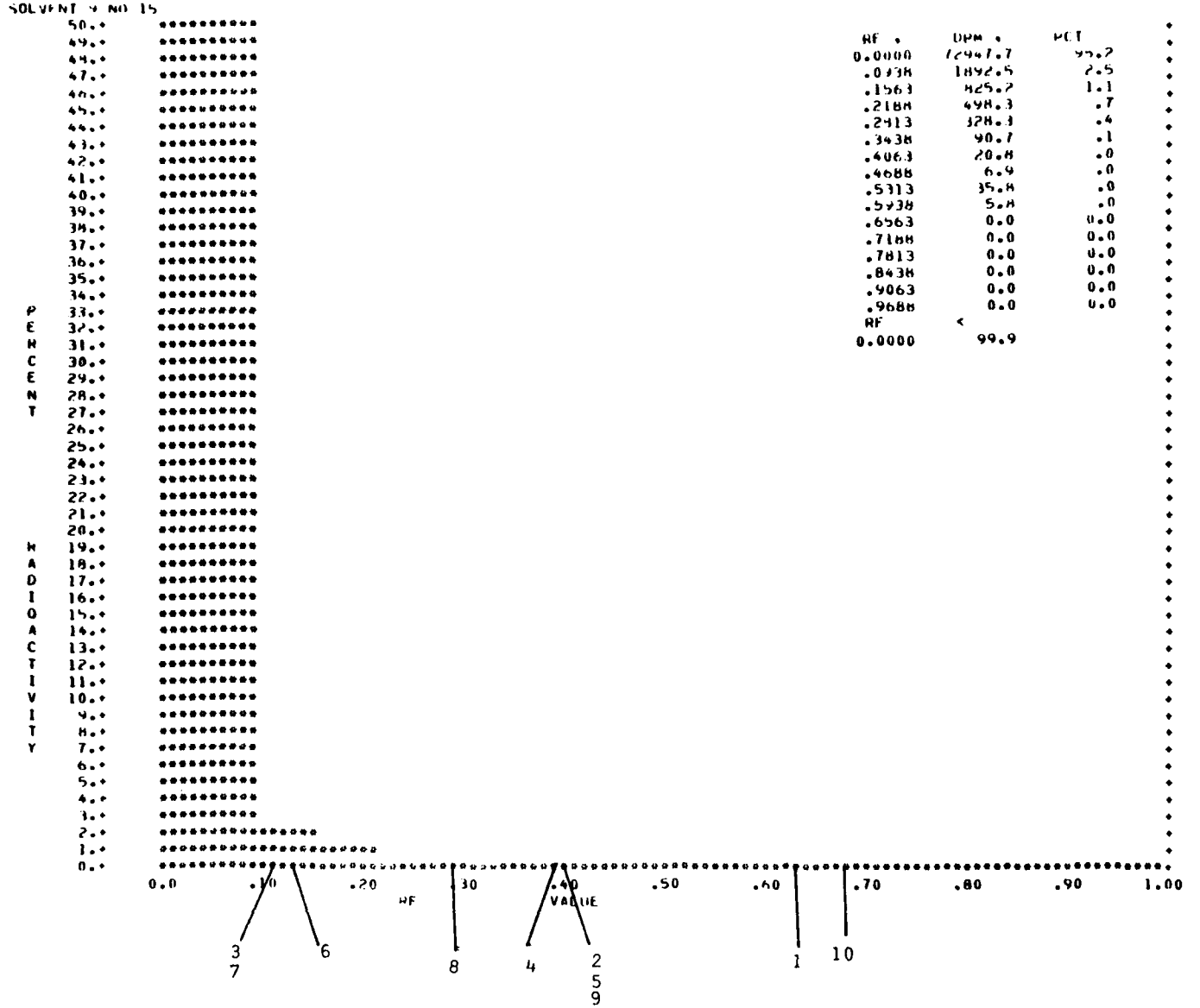


Figure 23-f-IX: Male Rabbits, Dermal Application, Solvent I

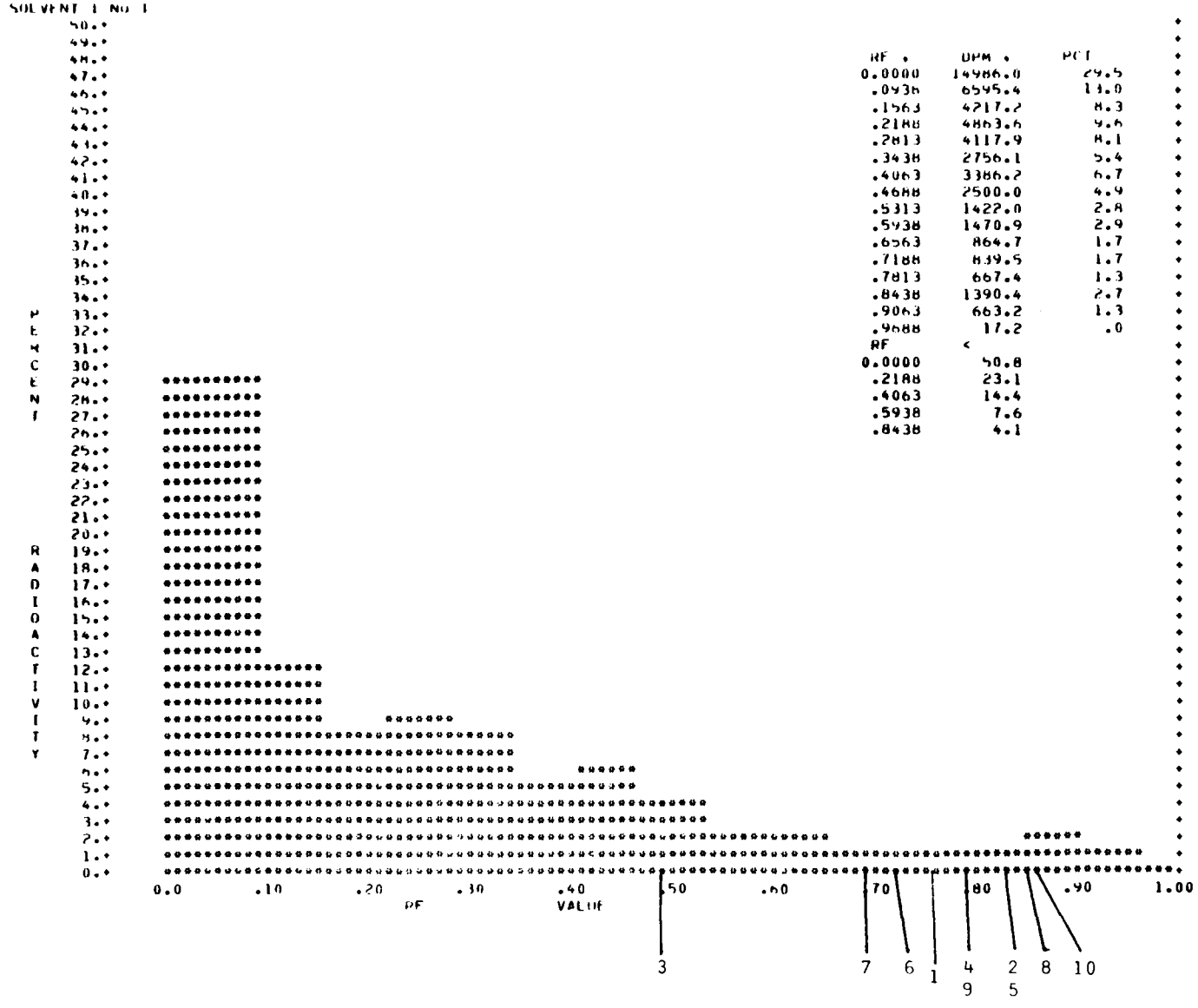


Figure 23-g-I: Male Dogs, Oral Treatment, Solvent I

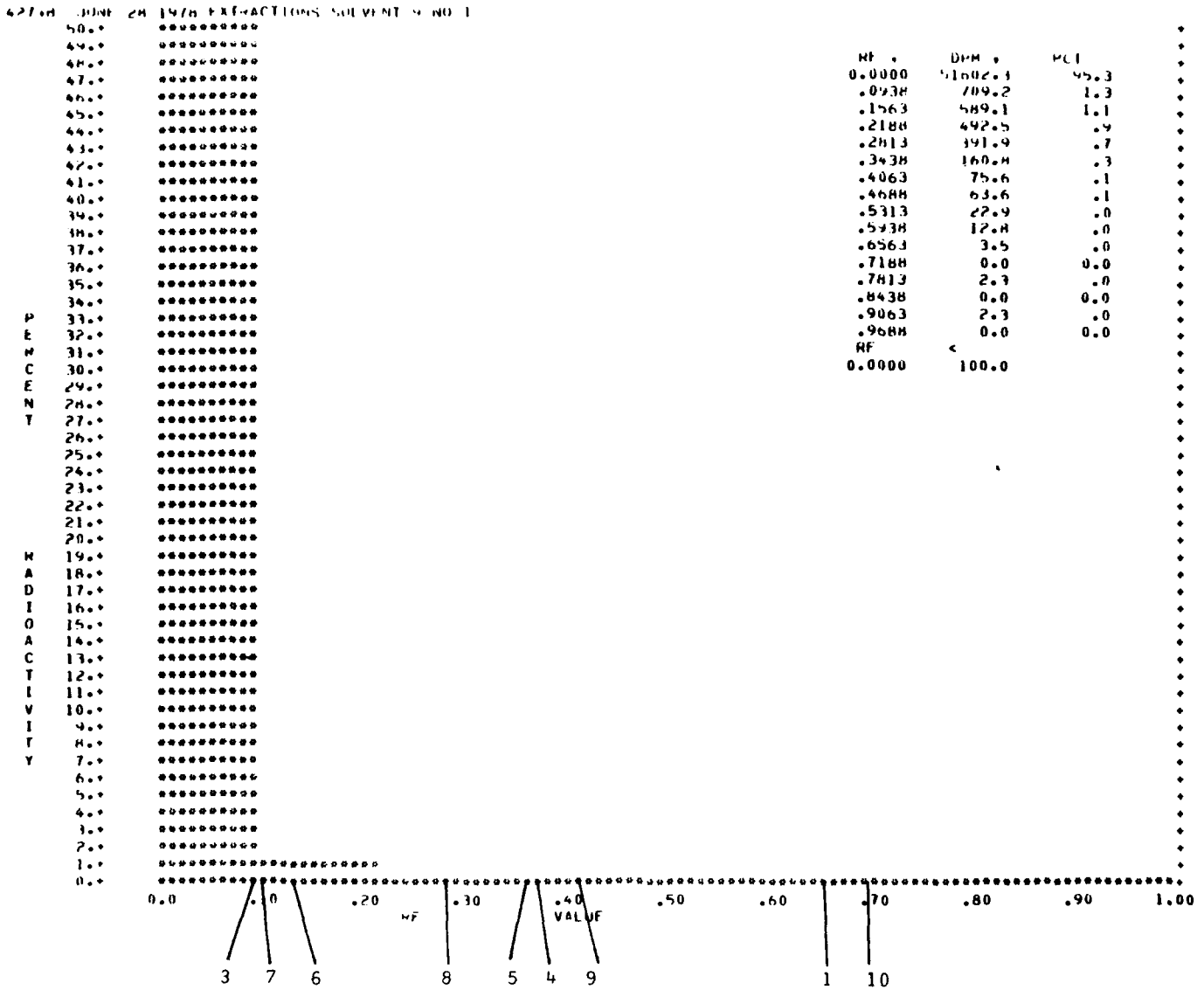


Figure 23-g-IX: Male Dogs, Oral Treatment, Solvent IX

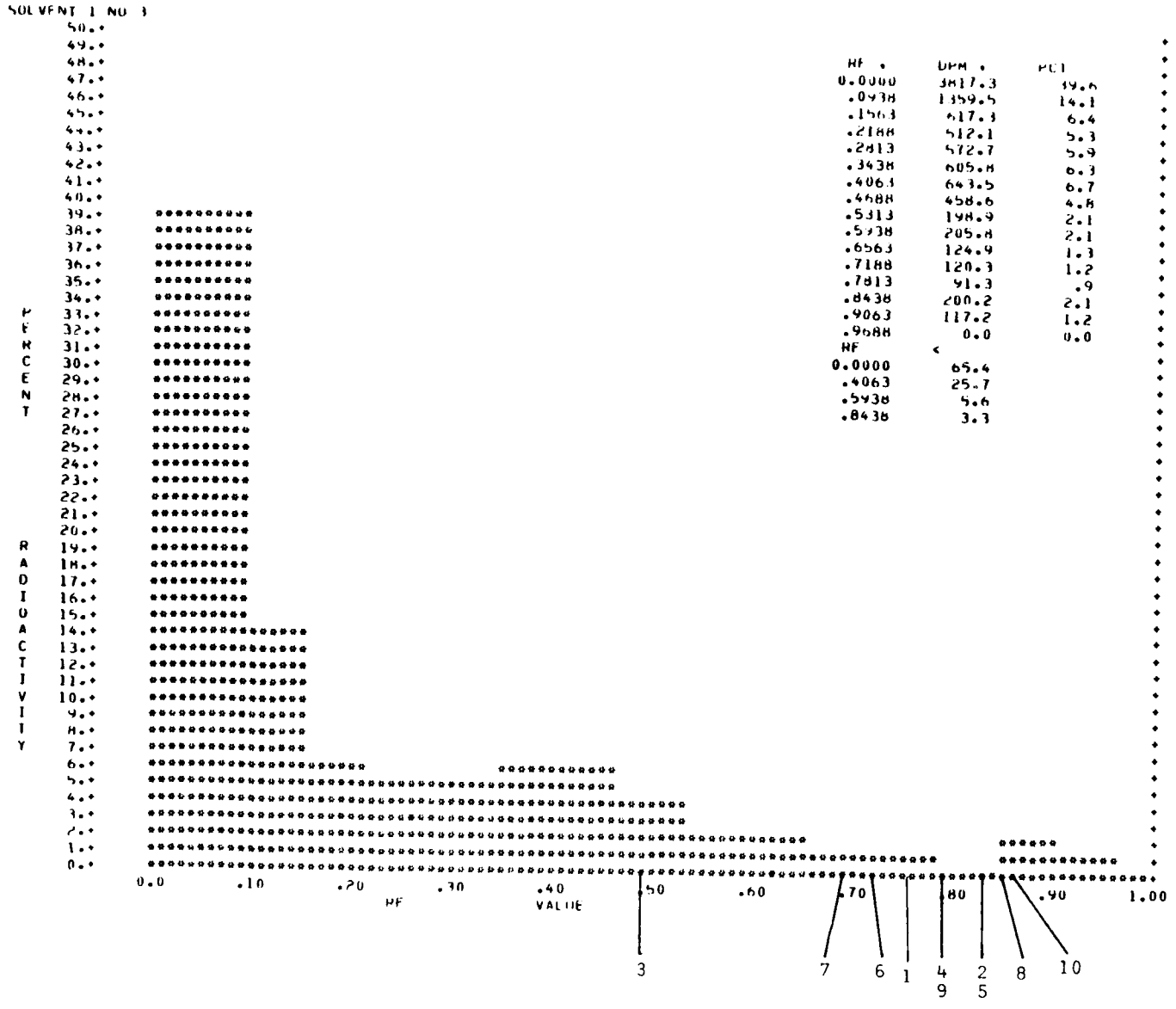


Figure 23-h-I: Male Dogs, Dermal Application, Solvent I

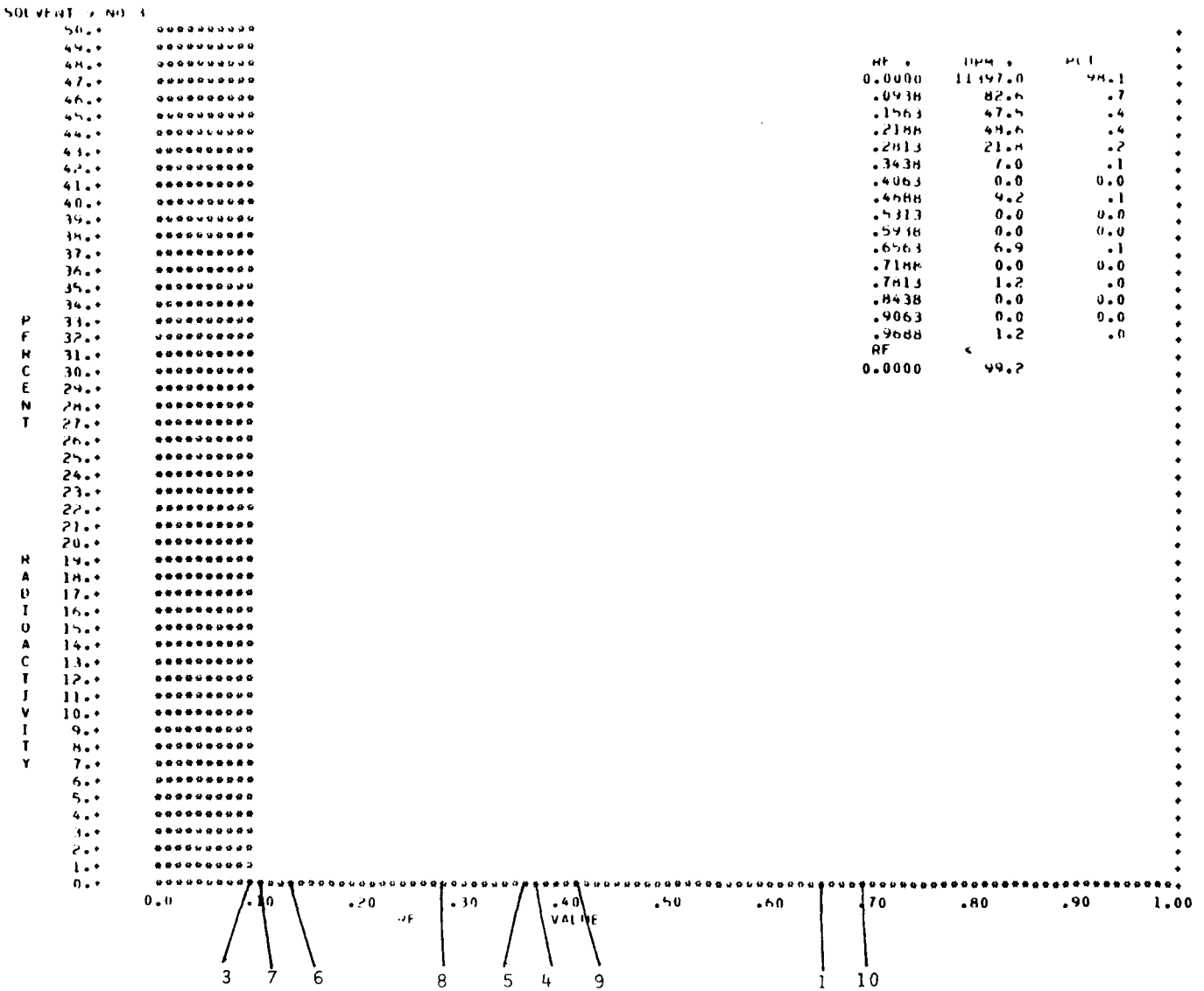


Figure 23-h-IX: Male Dogs, Dermal Application, Solvent IX

Figure 24: TLC of the Ethyl Acetate Extractable and Non-Extractable Material Obtained from Bile of Rabbits and Dogs Treated Orally or Dermally with ^{14}C-TNT. Reference standards are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzylalcohol
3. Trinitrobenzoic Acid
4. 4-Amino-2,6-Dinitrotoluene
5. 2-Amino-4,6-Dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2,6'-Tetranitro-4,4'-azoxytoluene

Figure 24 follows

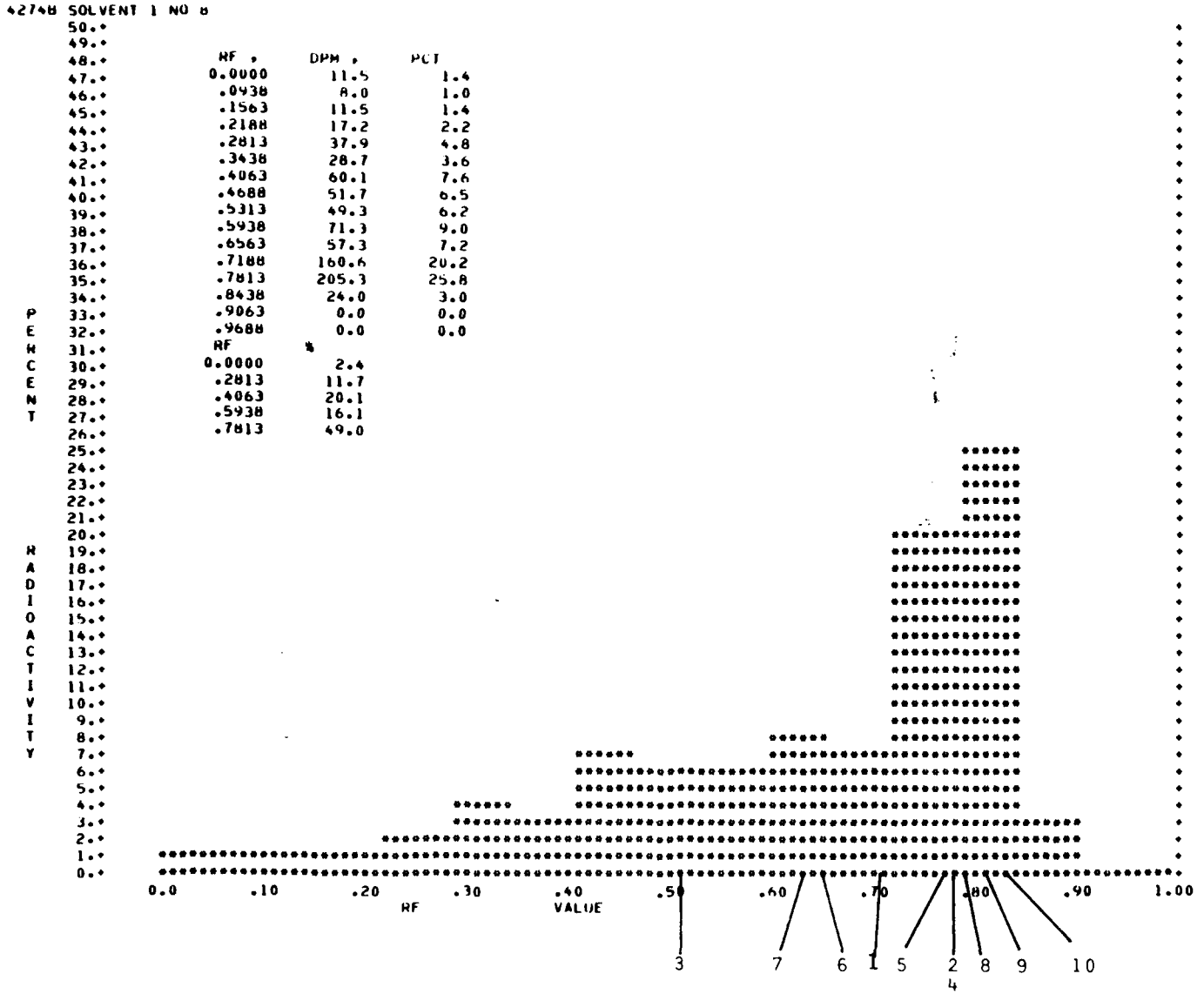


Figure 24-a-I: Rabbit, Oral Treatment, Ethyl Acetate Extract, Solvent I

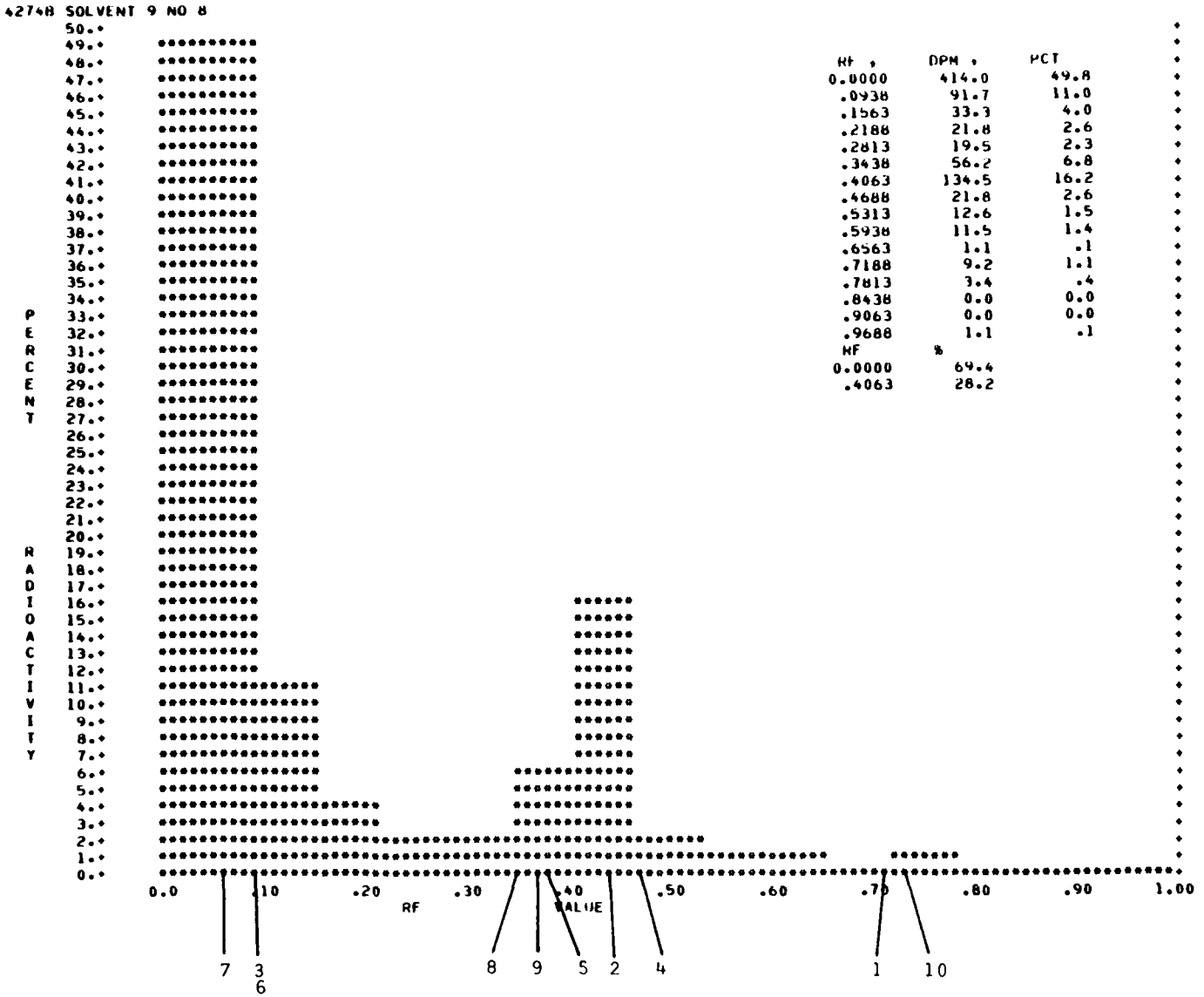


Figure 24-a-IX: Rabbit, Oral Treatment, Ethyl Acetate Extract, Solvent IX

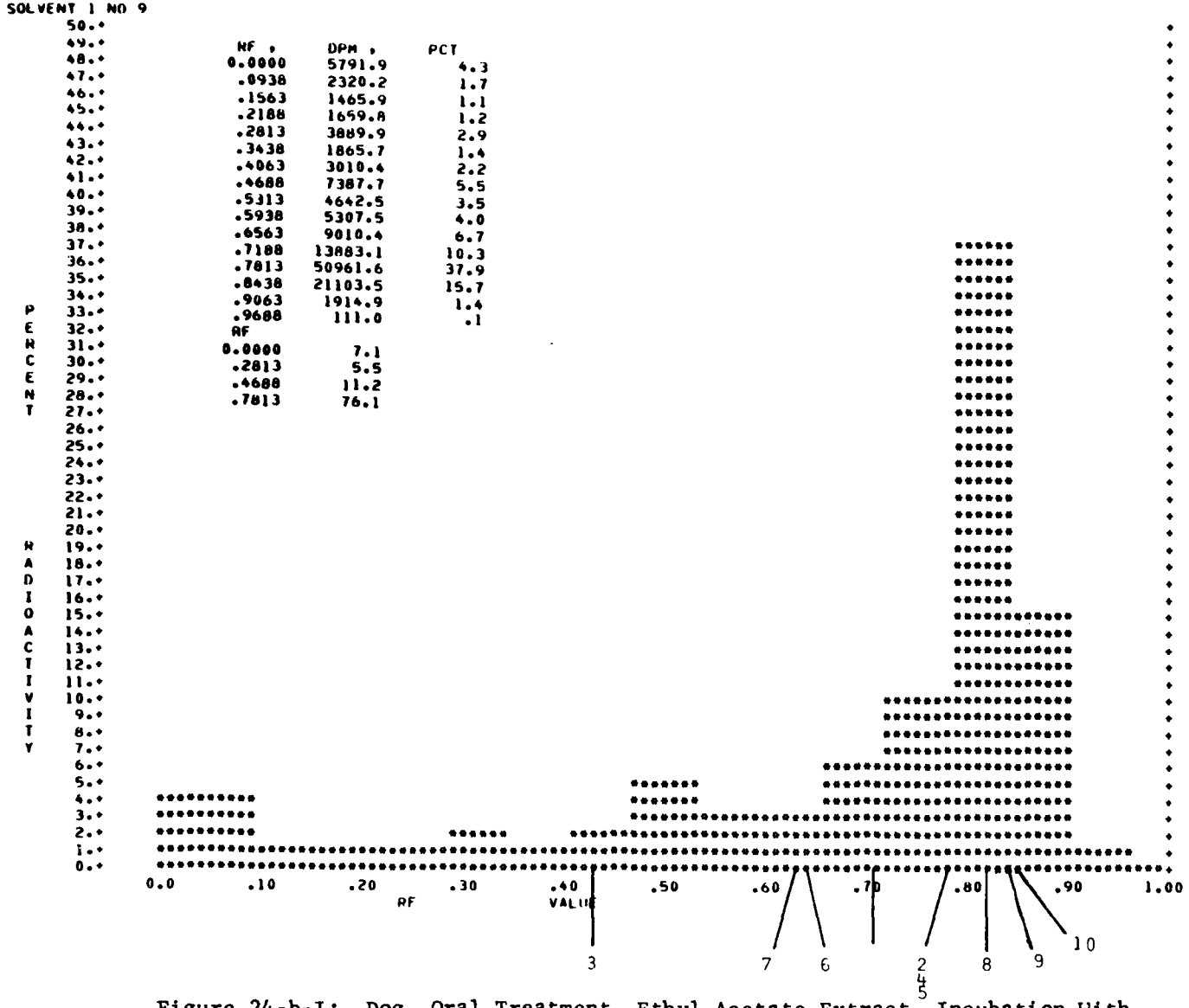


Figure 24-b-I: Dog, Oral Treatment, Ethyl Acetate Extract, Incubation With Water, Solvent I

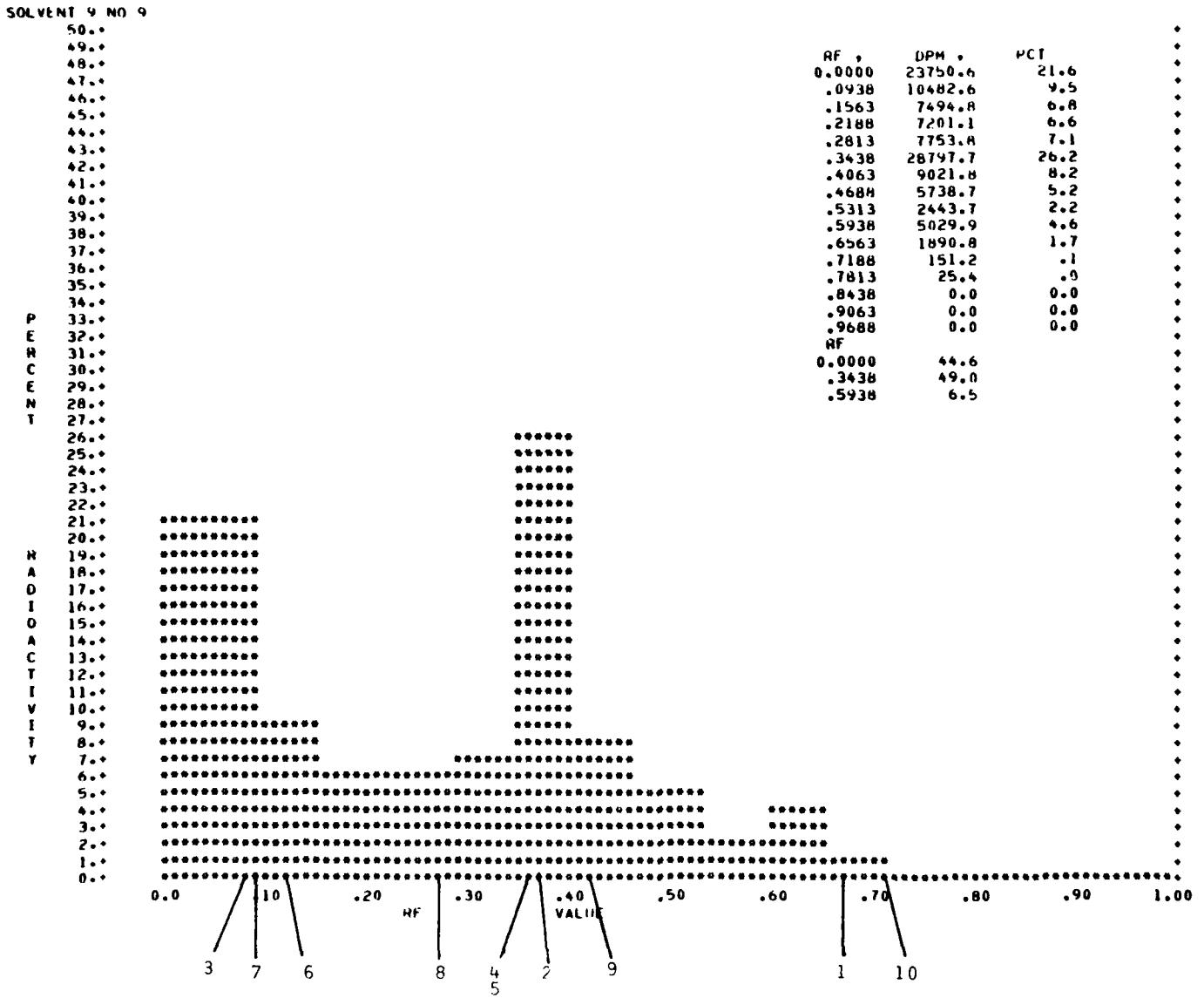


Figure 24-b-IX: Dog, Oral Treatment, Ethyl Acetate Extract, Incubation With Water, Solvent IX

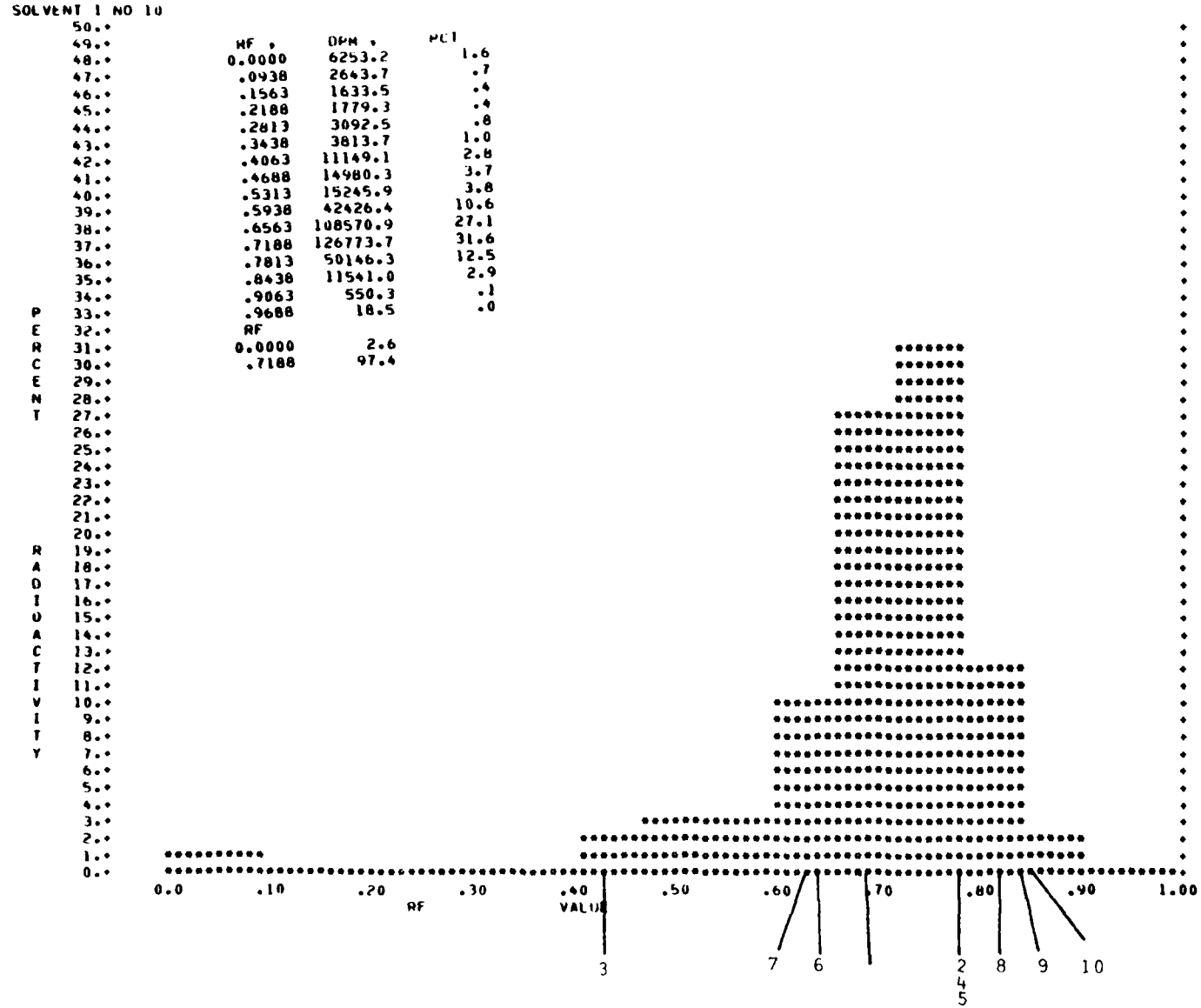


Figure 24-c-I: Dog, Oral Treatment, Ethyl Acetate Extract, Incubation With β-Glucuronidase, Solvent I

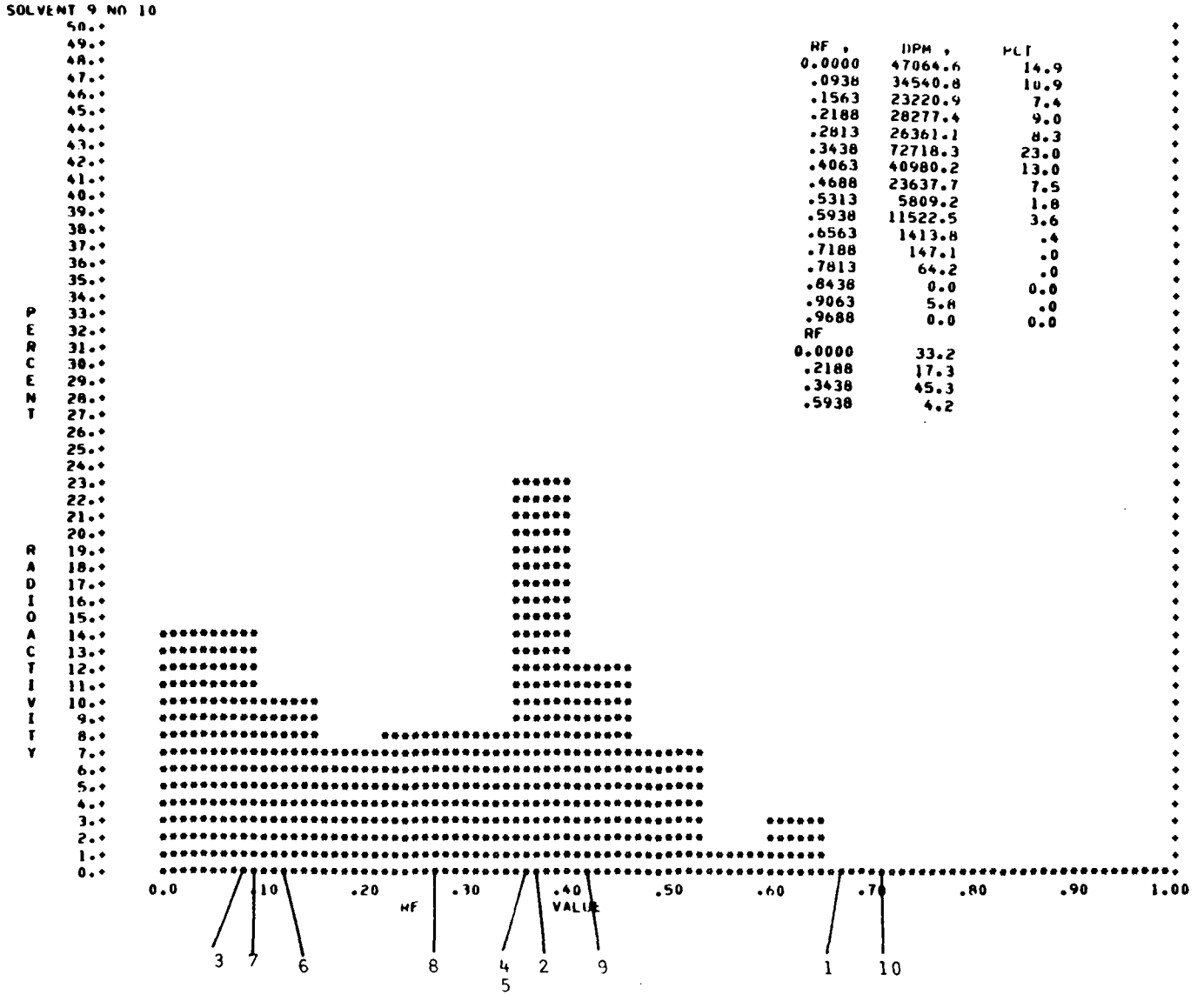


Figure 24-c-IX: Dog, Oral Treatment, Ethyl Acetate Extract, Incubation With β-Glucuronidase, Solvent IX

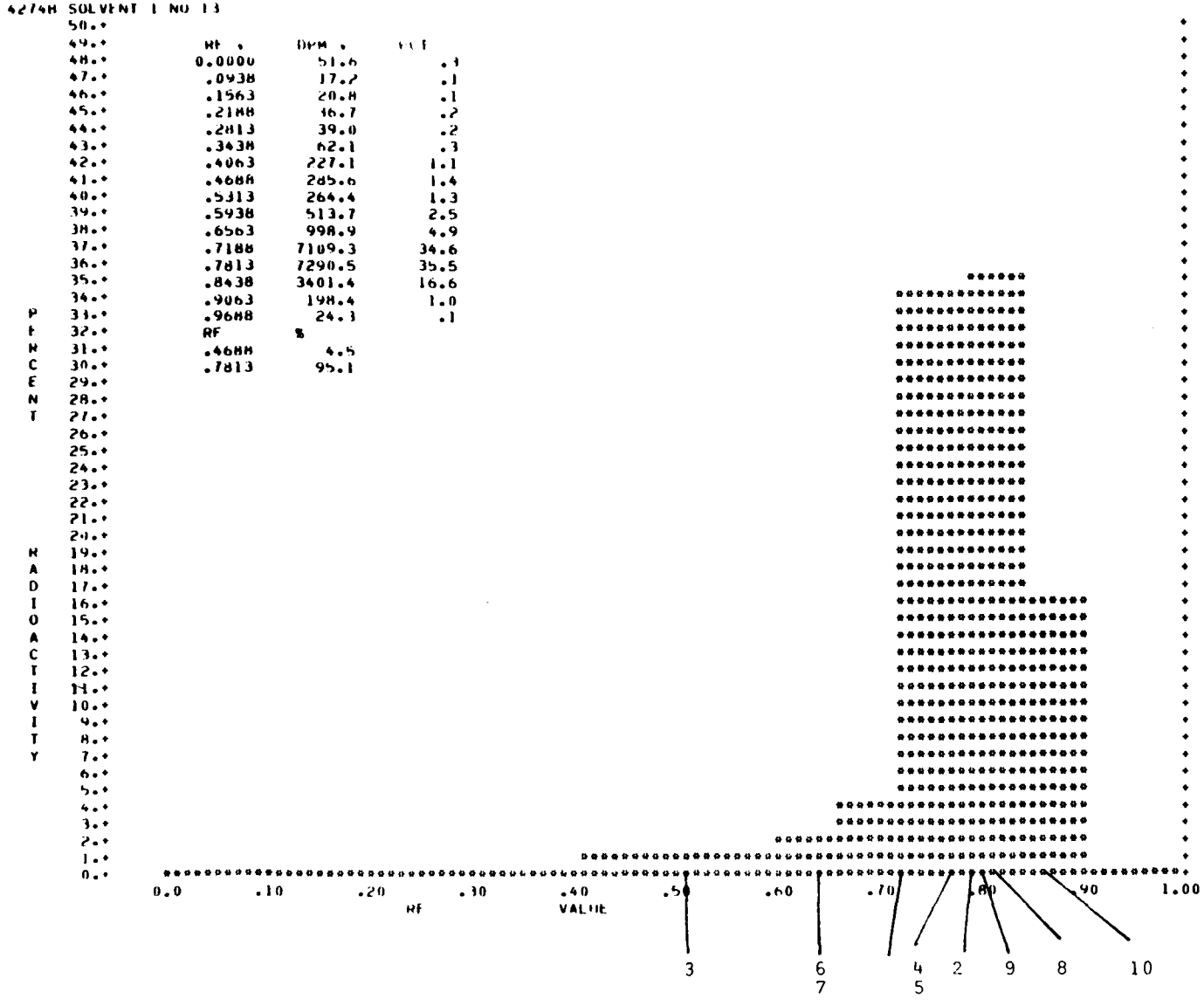


Figure 24-d-I: Dog, Dermal Application, Ethyl Acetate Extract, Incubation With Water, Solvent I

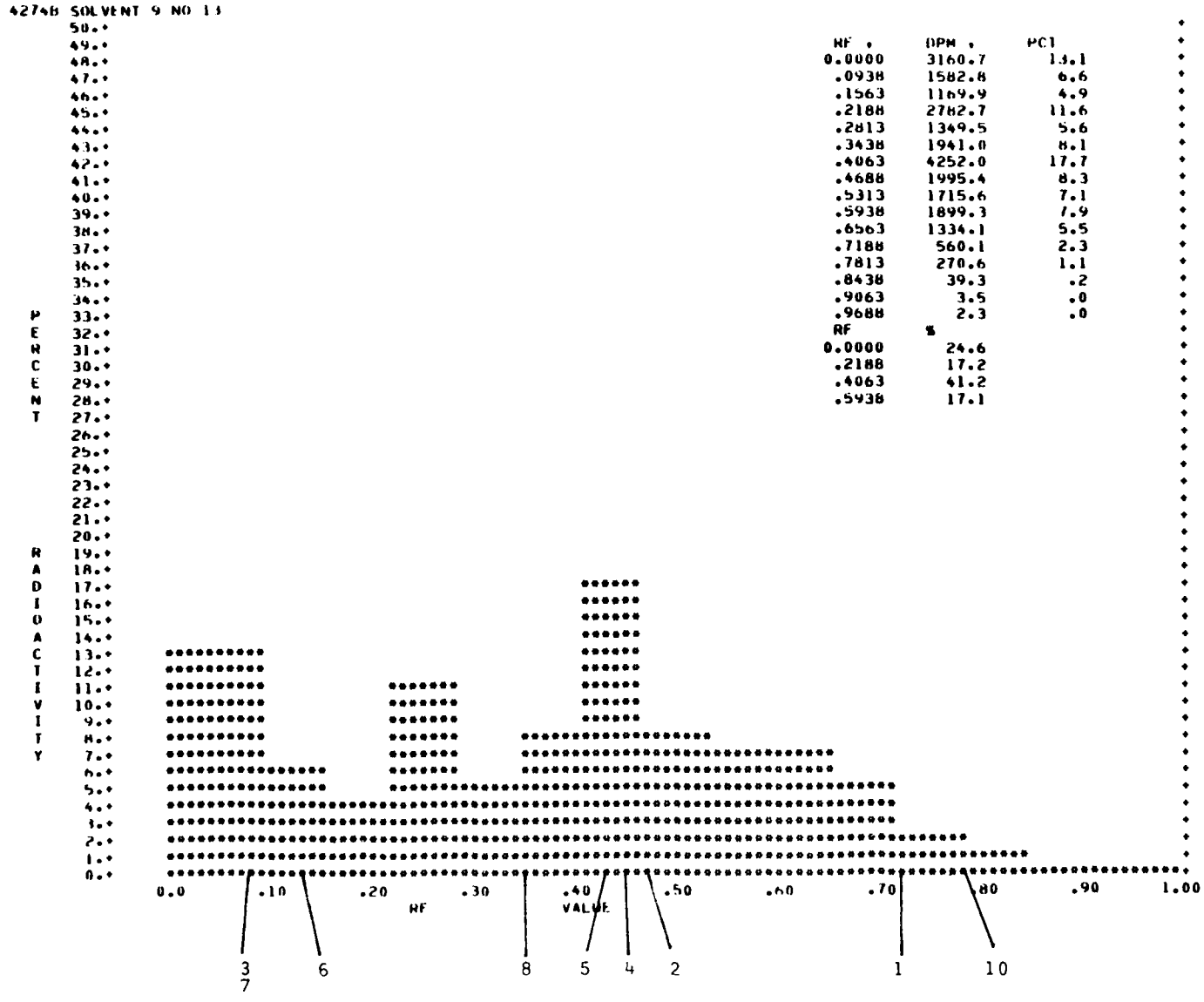


Figure 24-d-IX: Dog, Dermal Application, Ethyl Acetate Extract, Incubation With Water, Solvent IX

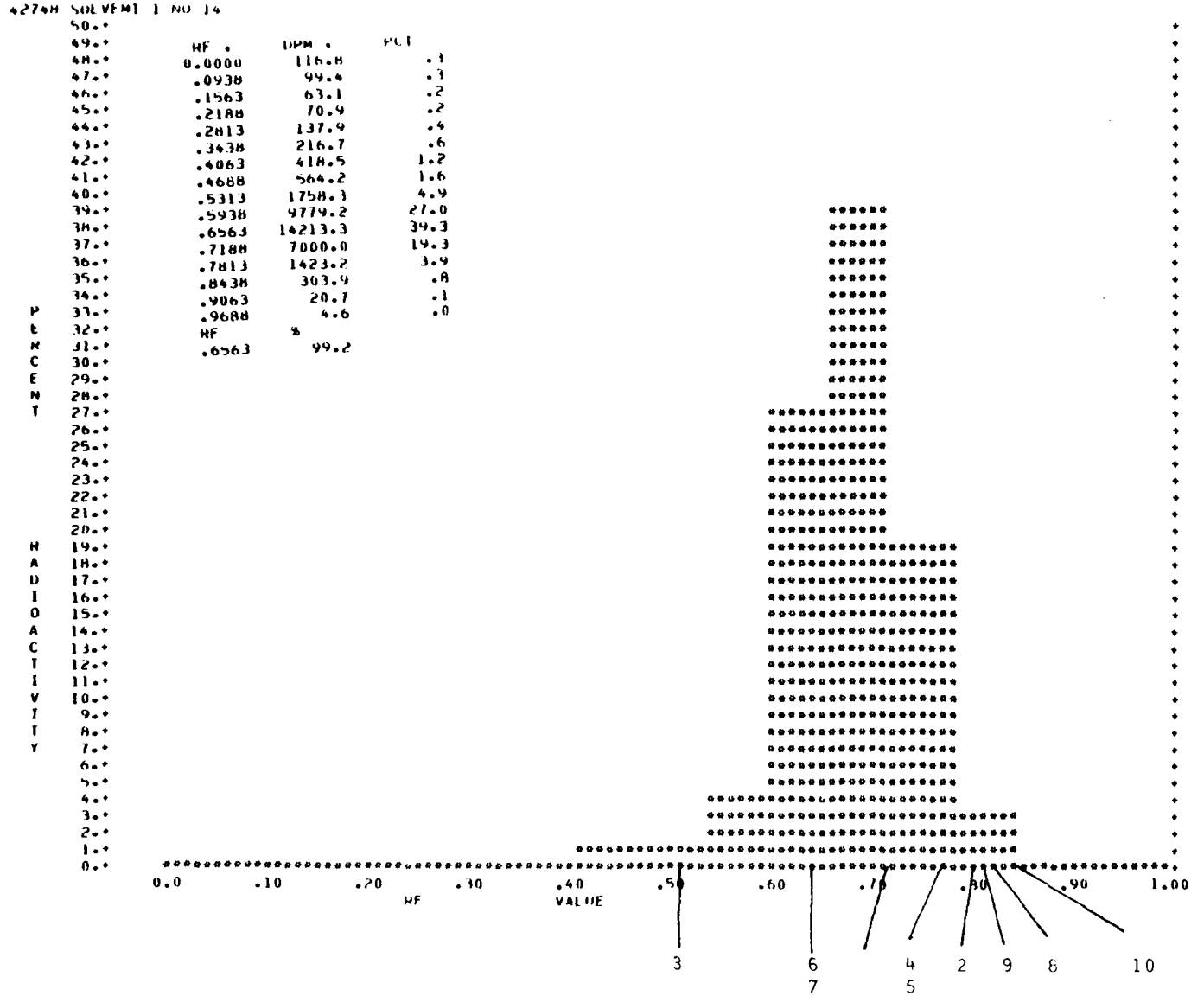


Figure 24-e-I: Dog, Dermal Application, Ethyl Acetate Extract, Incubation With β-Glucuronidase, Solvent I

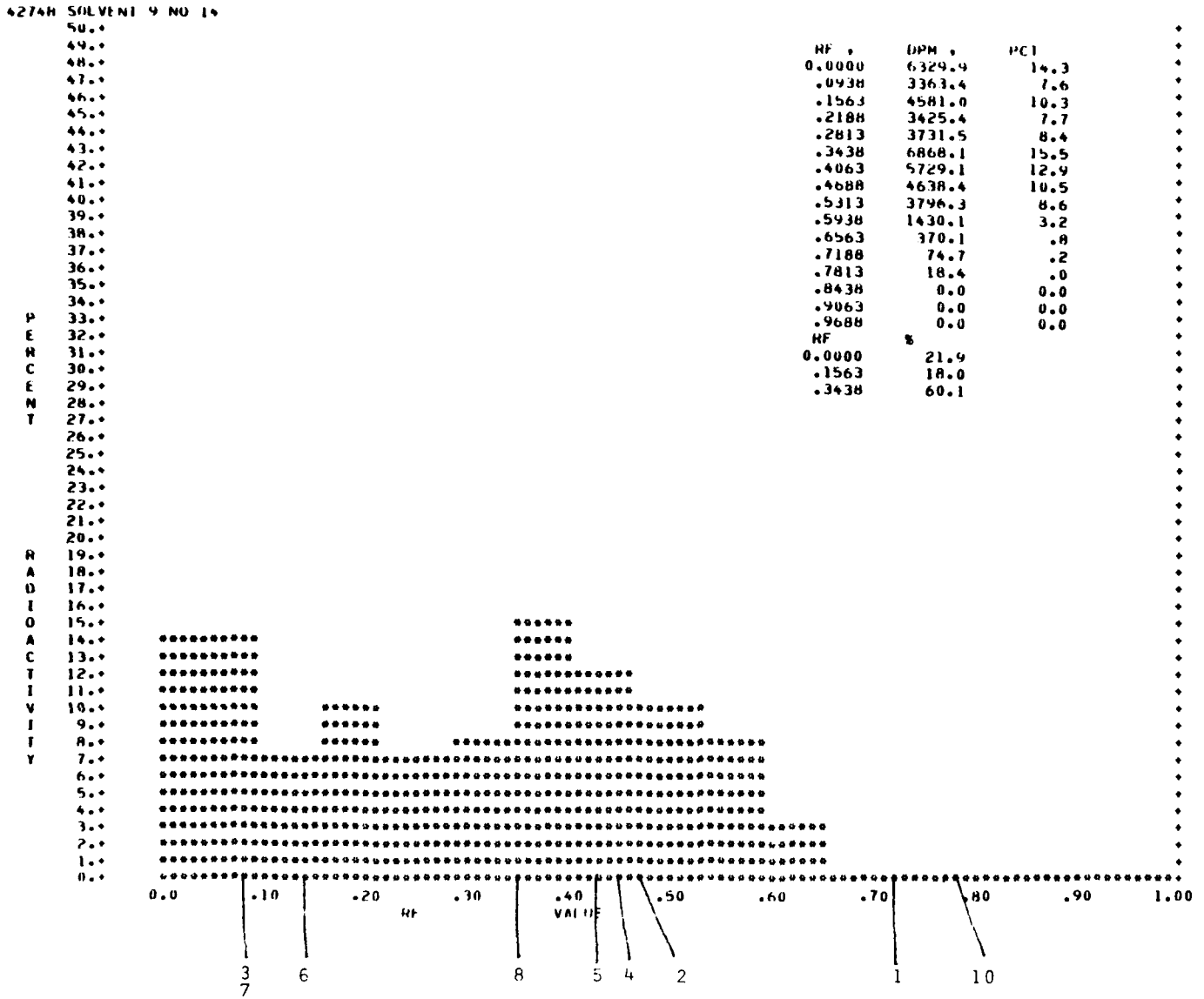


Figure 24-e-IX: Dog, Dermal Application, Ethyl Acetate Extract, Incubation With β-Glucuronidase, Solvent IX

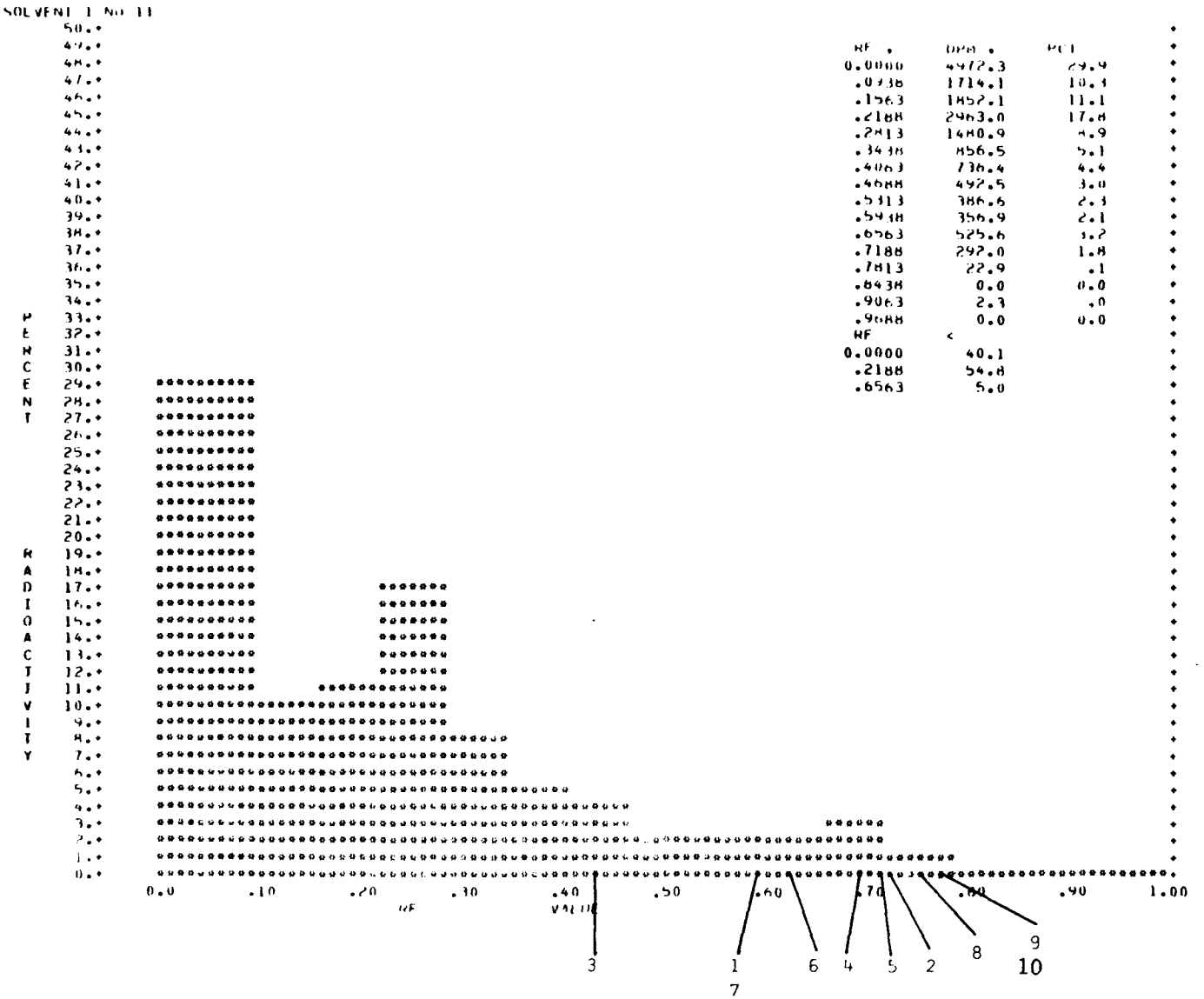
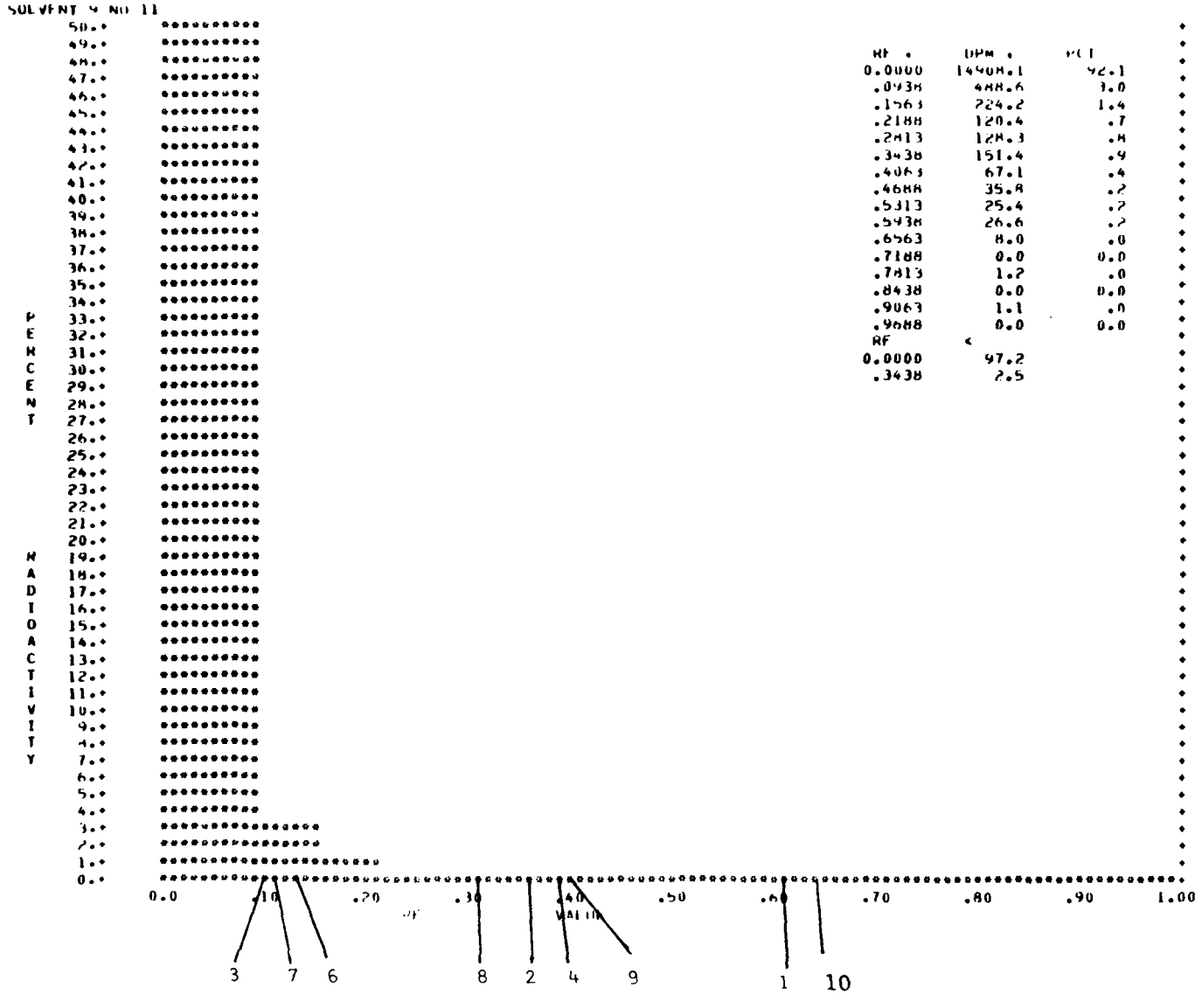


Figure 24-f-I: Dog, Dermal Application, Aqueous Extract, Solvent I



RF	DPM	PCT
0.0000	14908.1	92.1
.0938	488.6	3.0
.1563	224.2	1.4
.2188	120.4	.7
.2813	128.3	.8
.3438	151.4	.9
.4063	67.1	.4
.4688	35.8	.2
.5313	25.4	.2
.5938	26.6	.2
.6563	8.0	.0
.7188	0.0	0.0
.7813	1.2	.0
.8438	0.0	0.0
.9063	1.1	.0
.9688	0.0	0.0

RF	<
0.0000	97.2
.3438	2.5

Figure 24-f-IX: Dog, Dermal Application, Aqueous Extract, Solvent IX

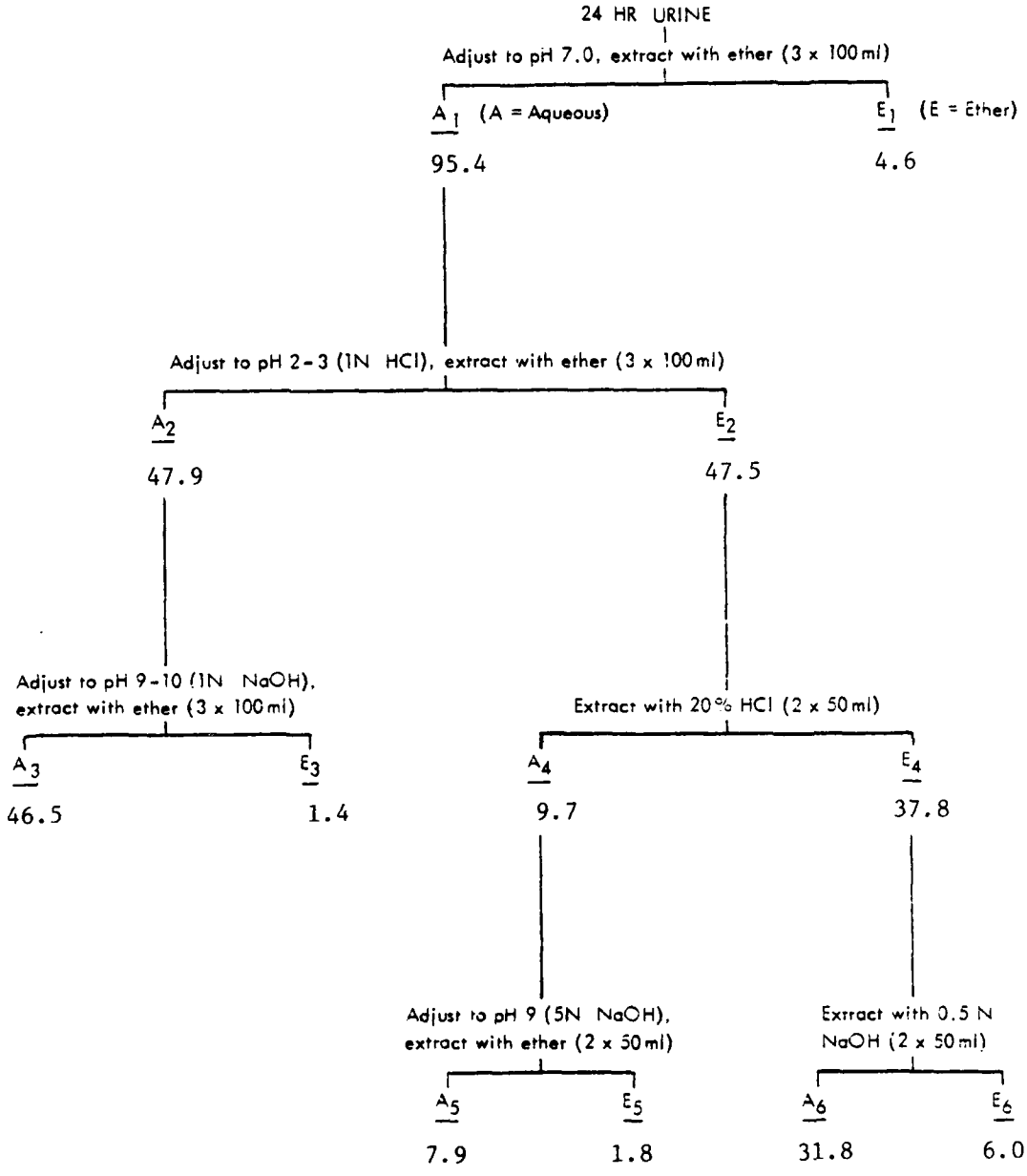


Figure 25: Fractionation of 24-Hr Urine Obtained from Rats Treated Orally with ^{14}C-TNT. Values indicate the percentage of extractable radioactivity in each fraction.

Figure 26, E_1-E_6: TLC of Ether-Extractable Products Obtained from 24-hr Urine of Rats Treated Orally with ^{14}C-TNT. Extractions were performed at different pH conditions according to the scheme described in the preceding figure. Solvent I, n-butanol:acetic acid: water, 1:1:1; Solvent IX, toluene:acetic acid, 4:1. Reference metab olites are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzyl alcohol
3. Trinitrobenzoic Acid
4. 4-Amino-2,6-dinitrotoluene
5. 2-Amino-4,6-dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2',6'-Tetranitro-4,4'-azoxytoluene

Figure 26 follows

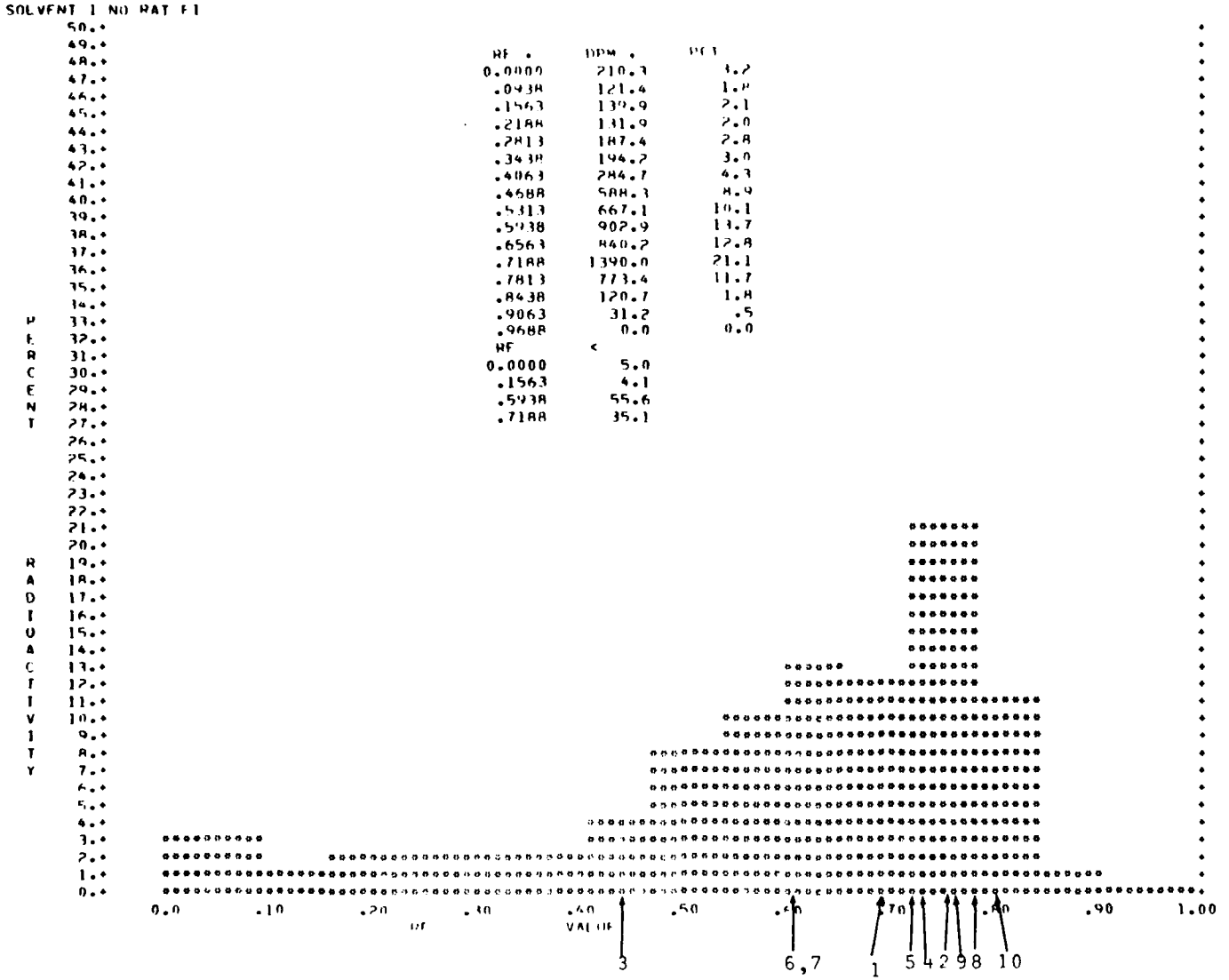


Figure 26-E_1: Solvent I

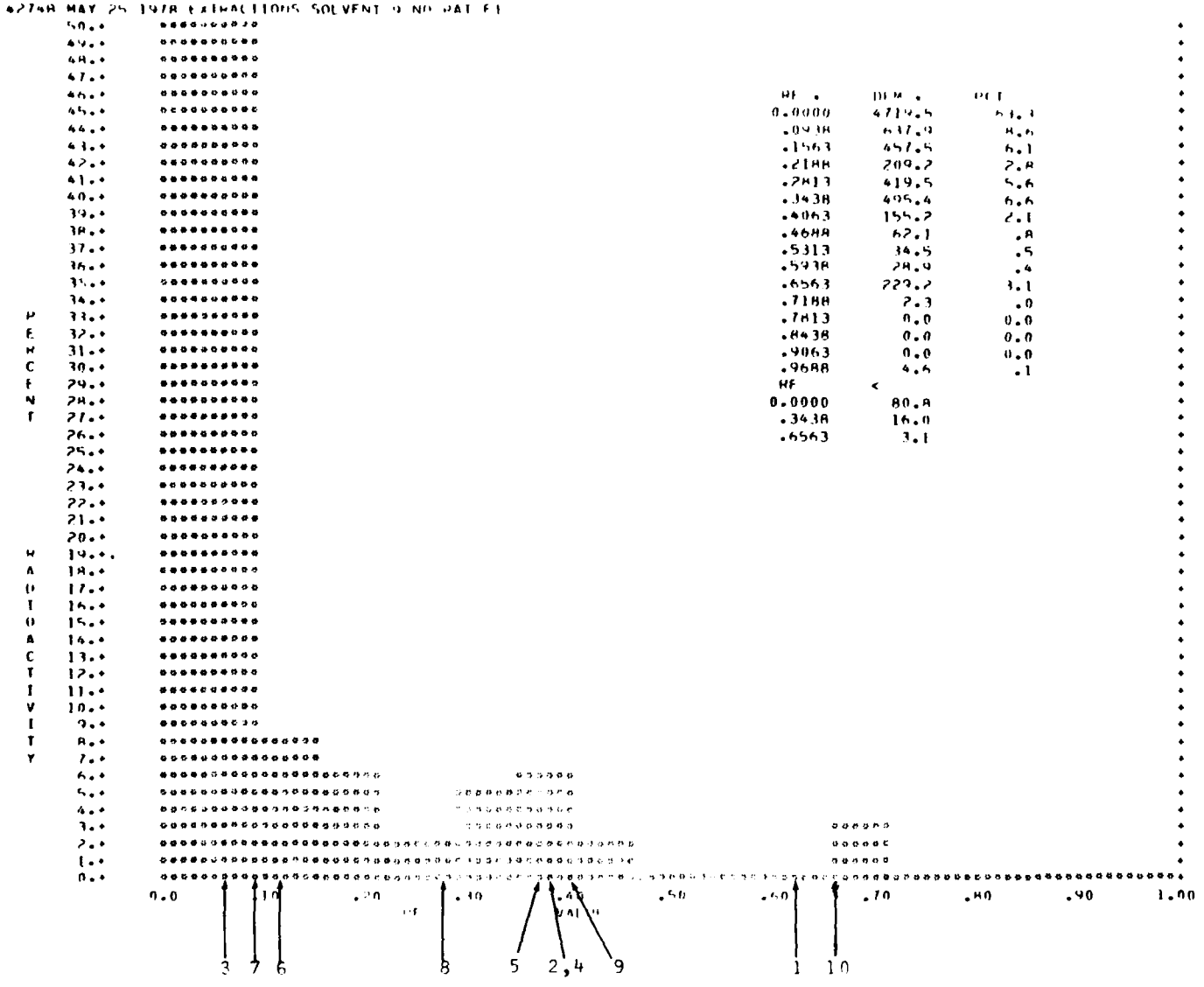


Figure 26-E_1: Solvent IX

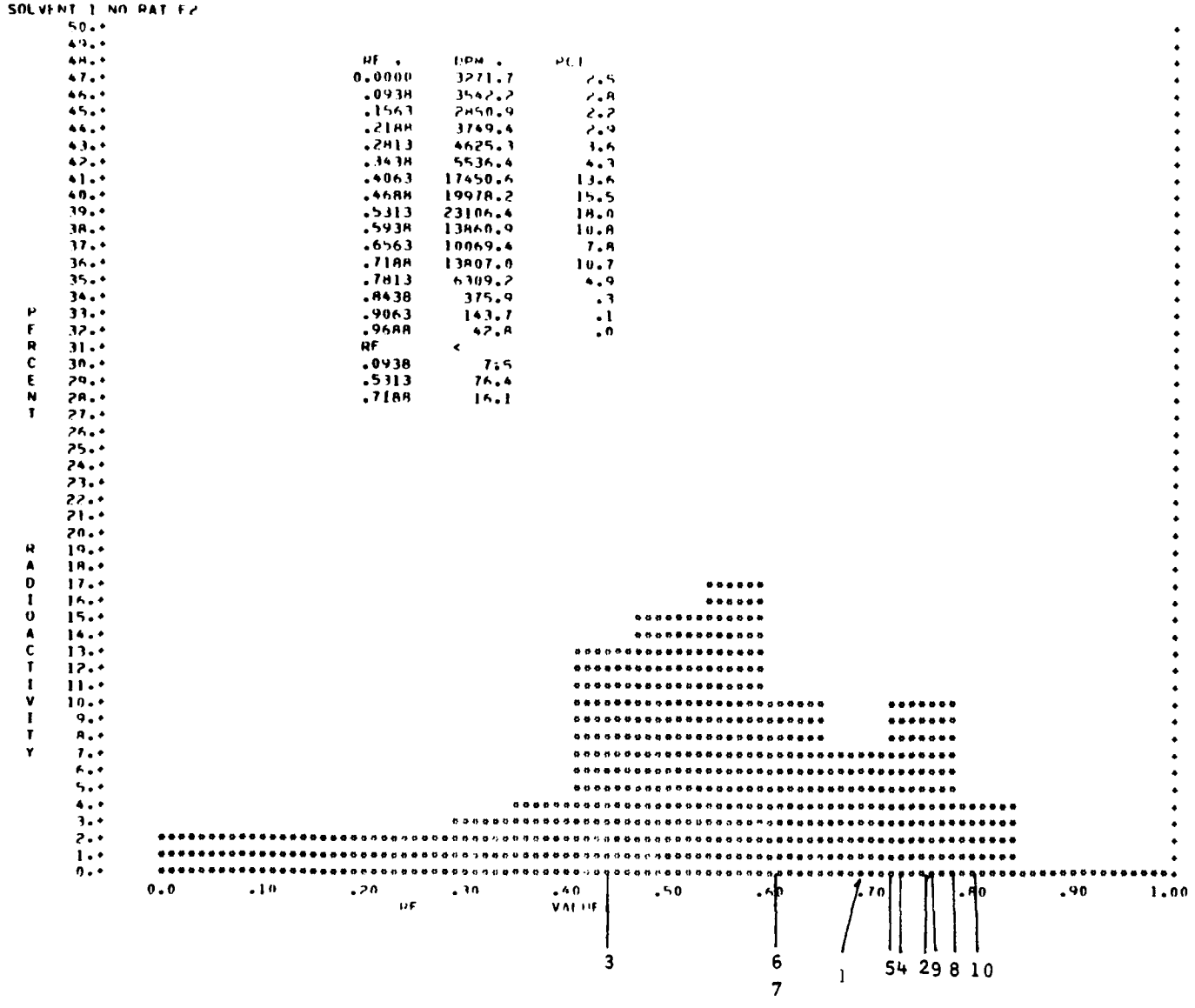


Figure 26-E_2: Solvent I

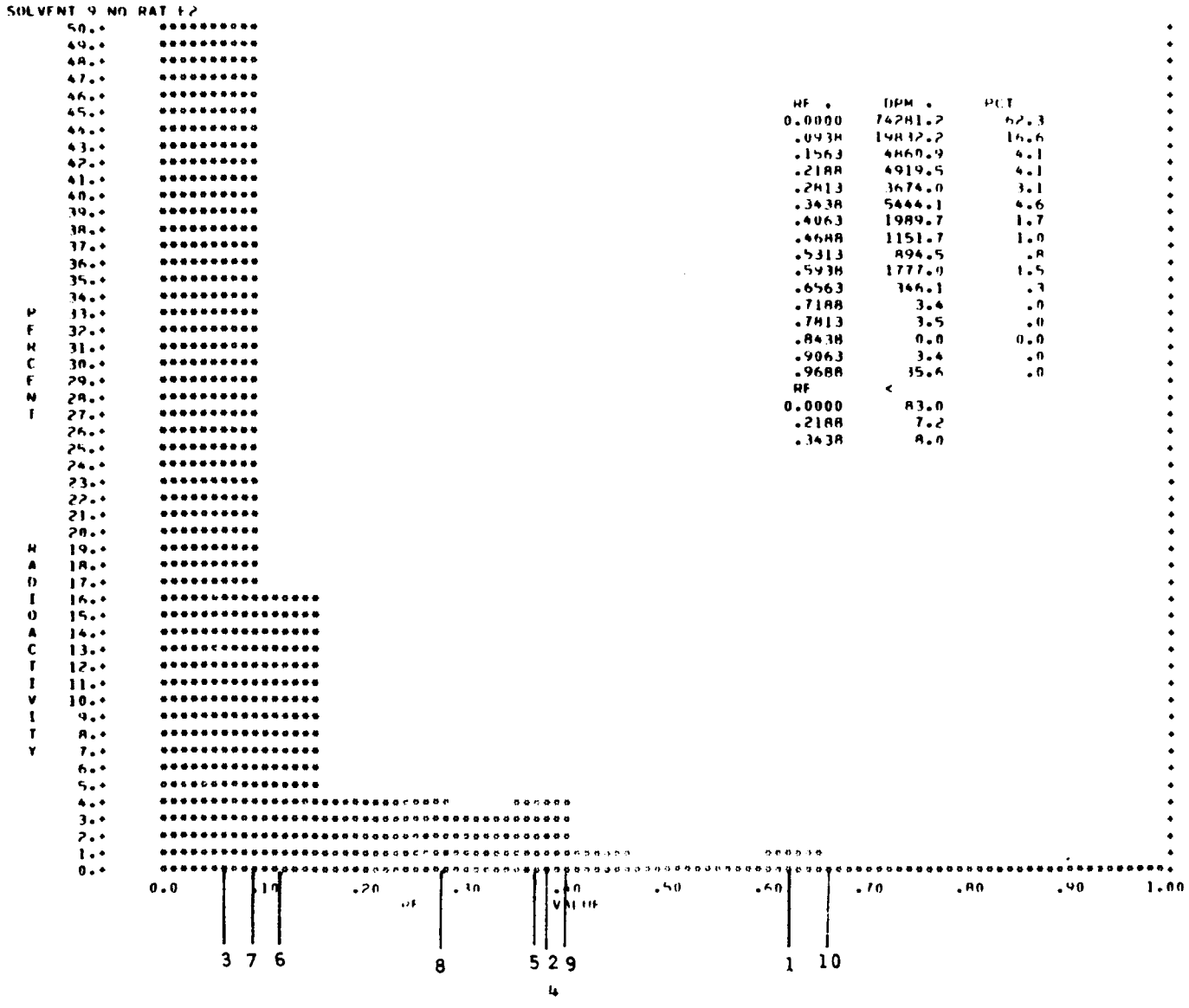


Figure 26-E_2: Solvent IX

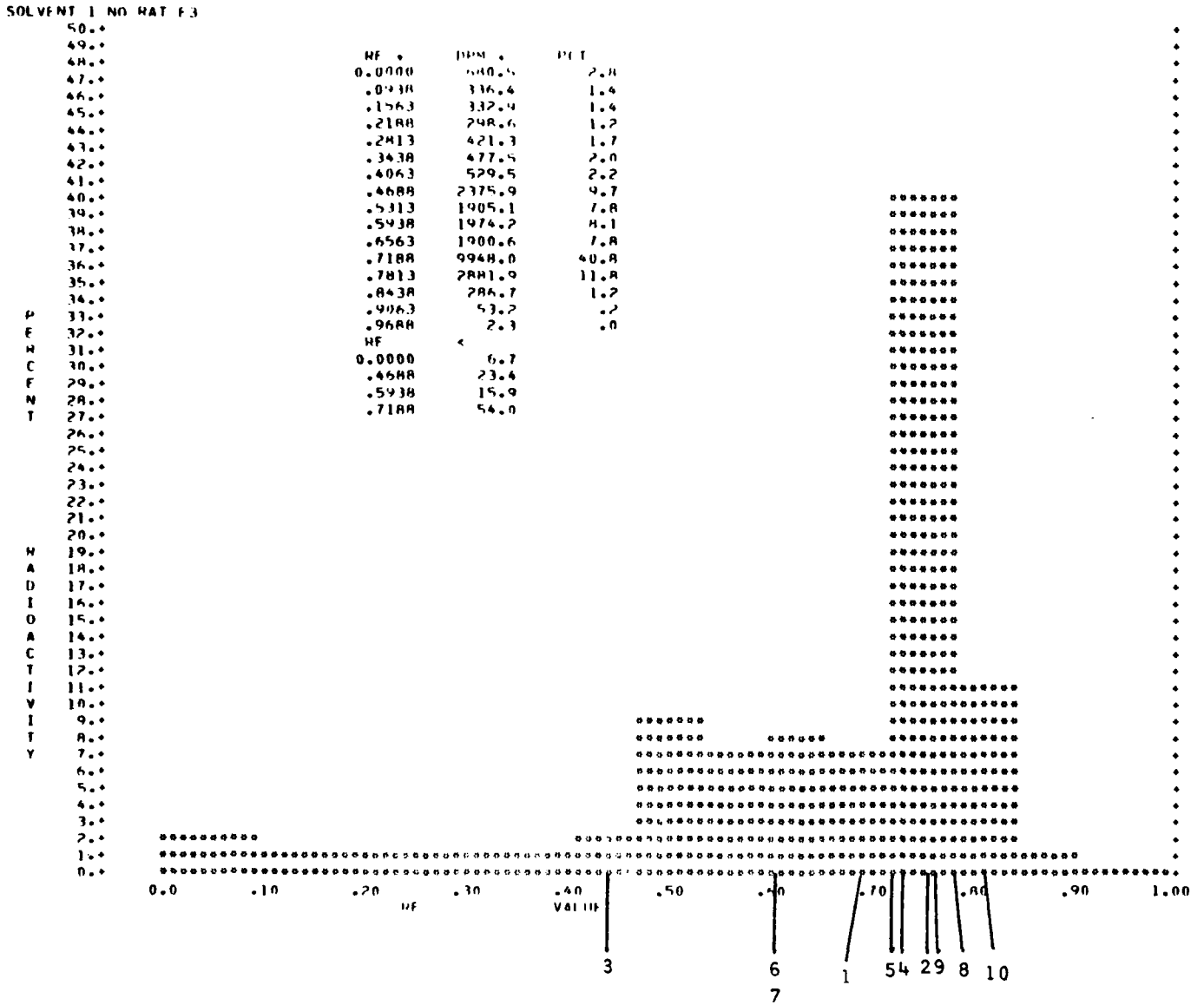


Figure 26-E3: Solvent I

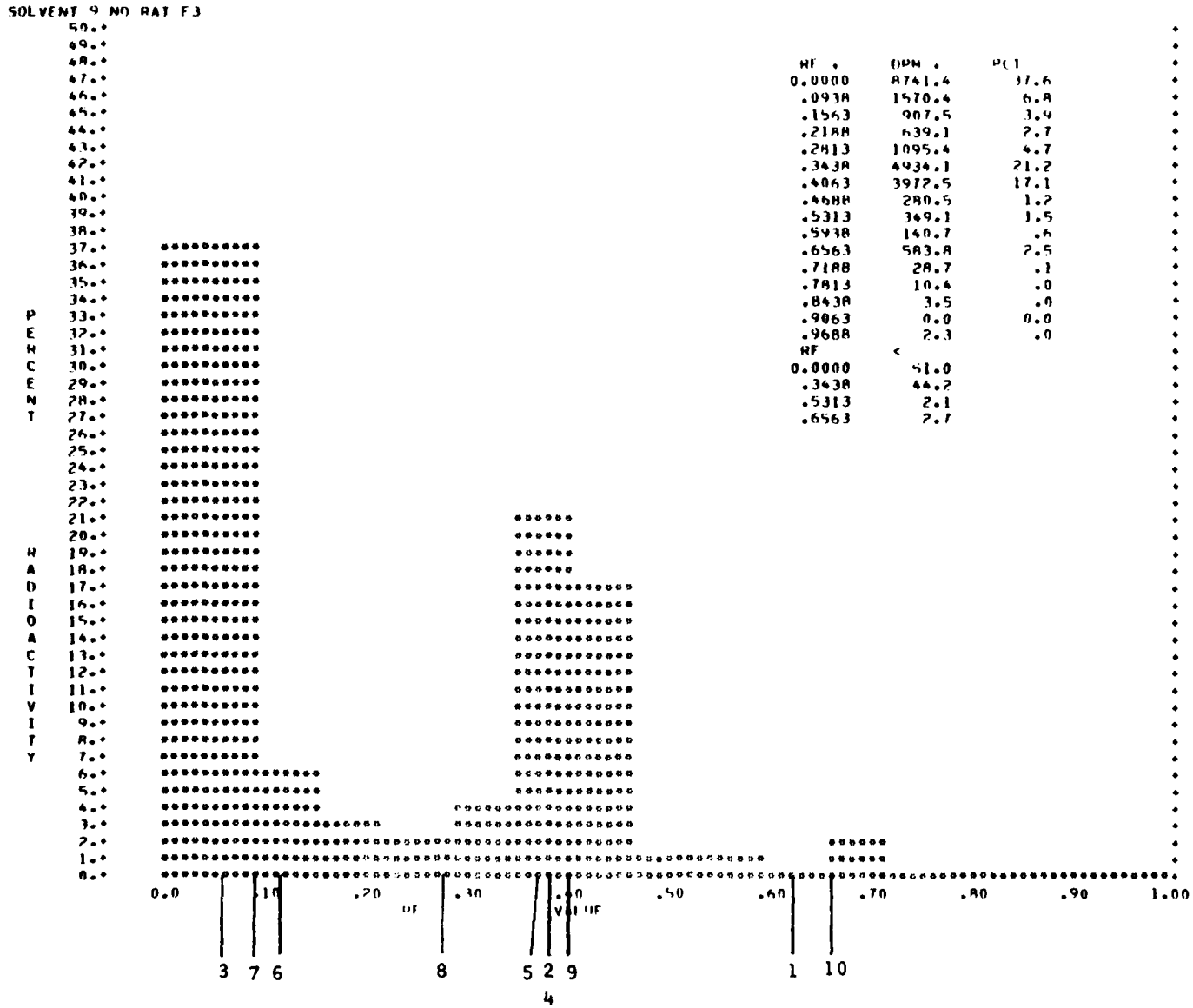


Figure 26-E_3: Solvent IX

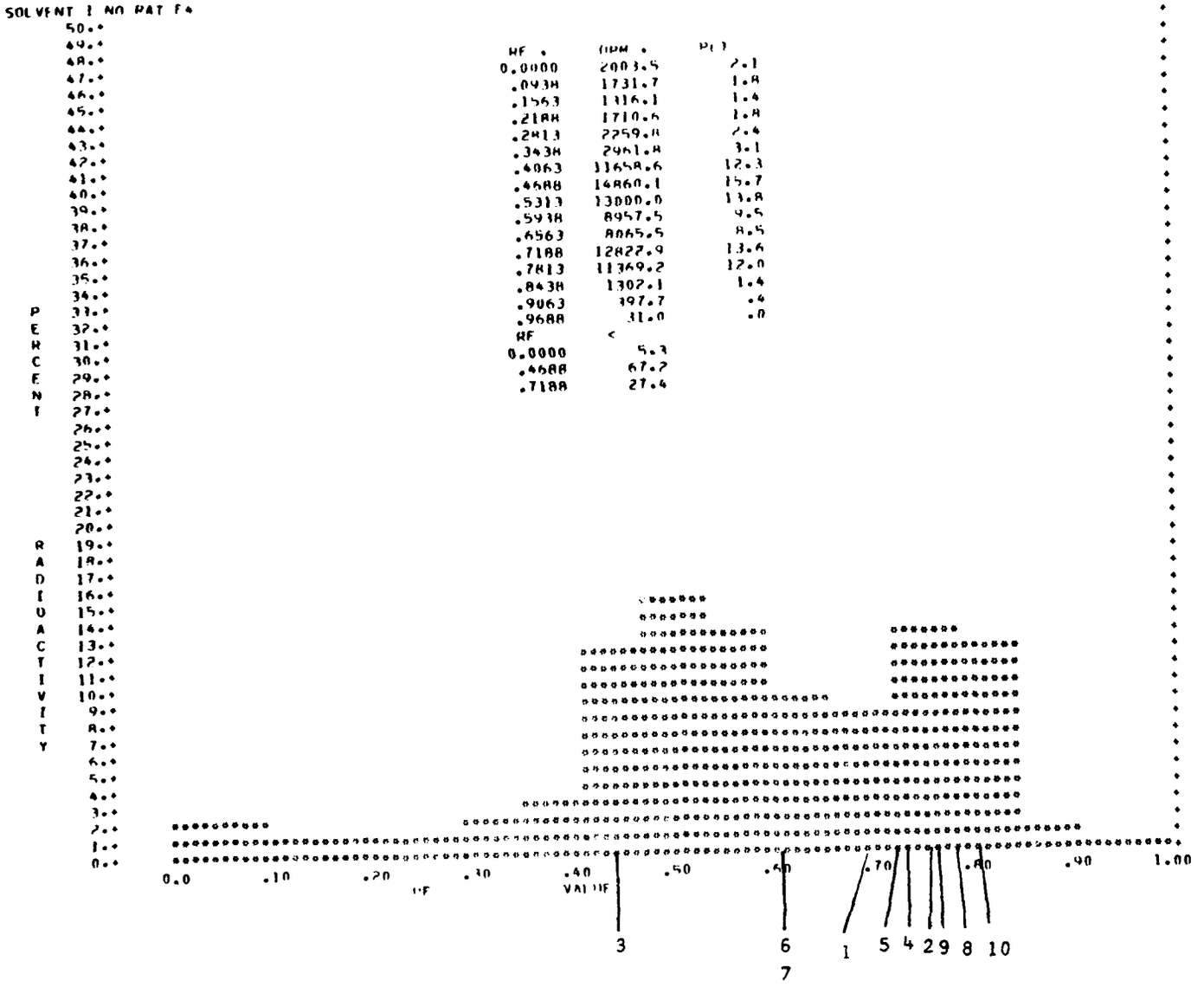


Figure 26-E_4: Solvent I

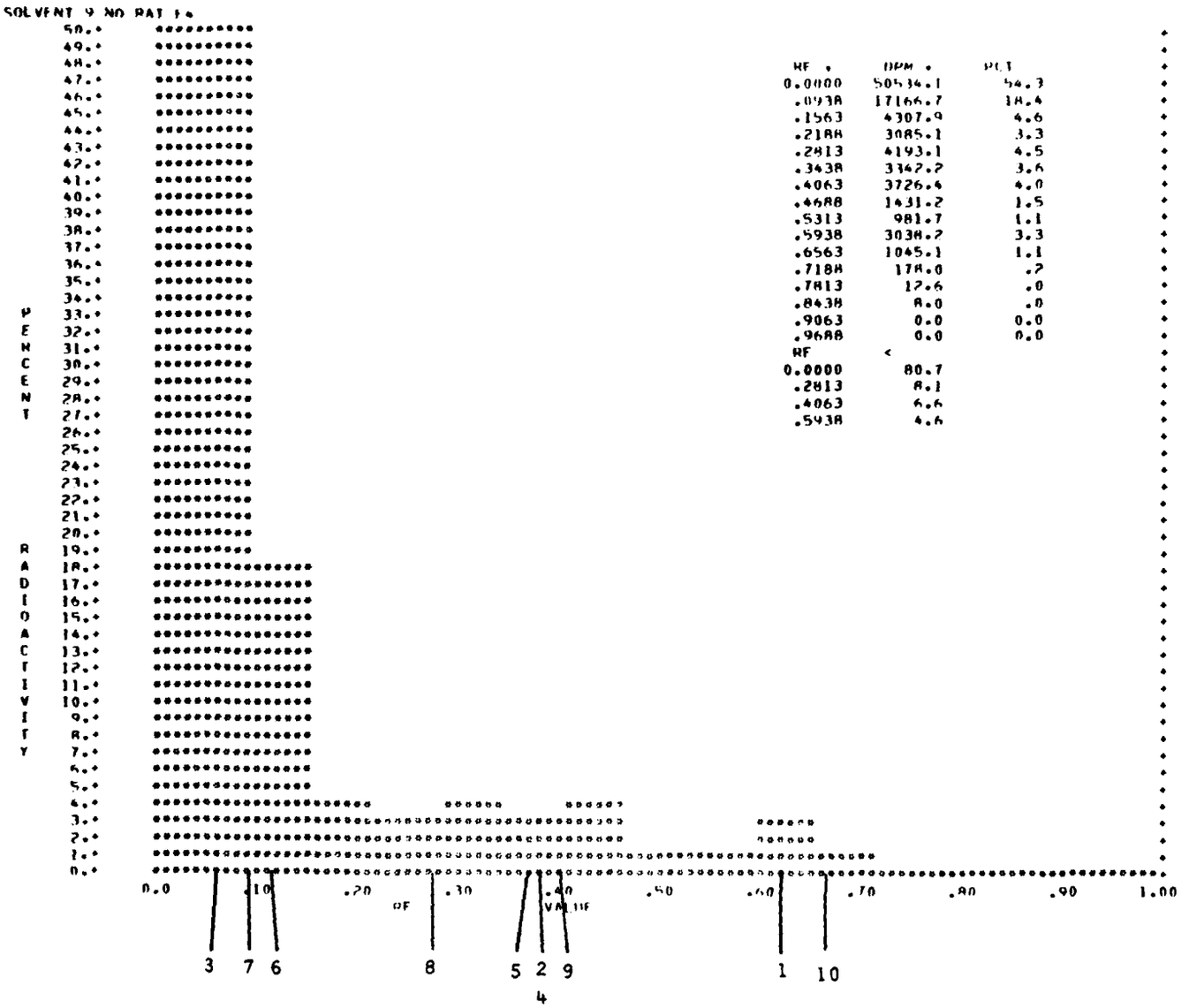


Figure 26-E_4: Solvent IX

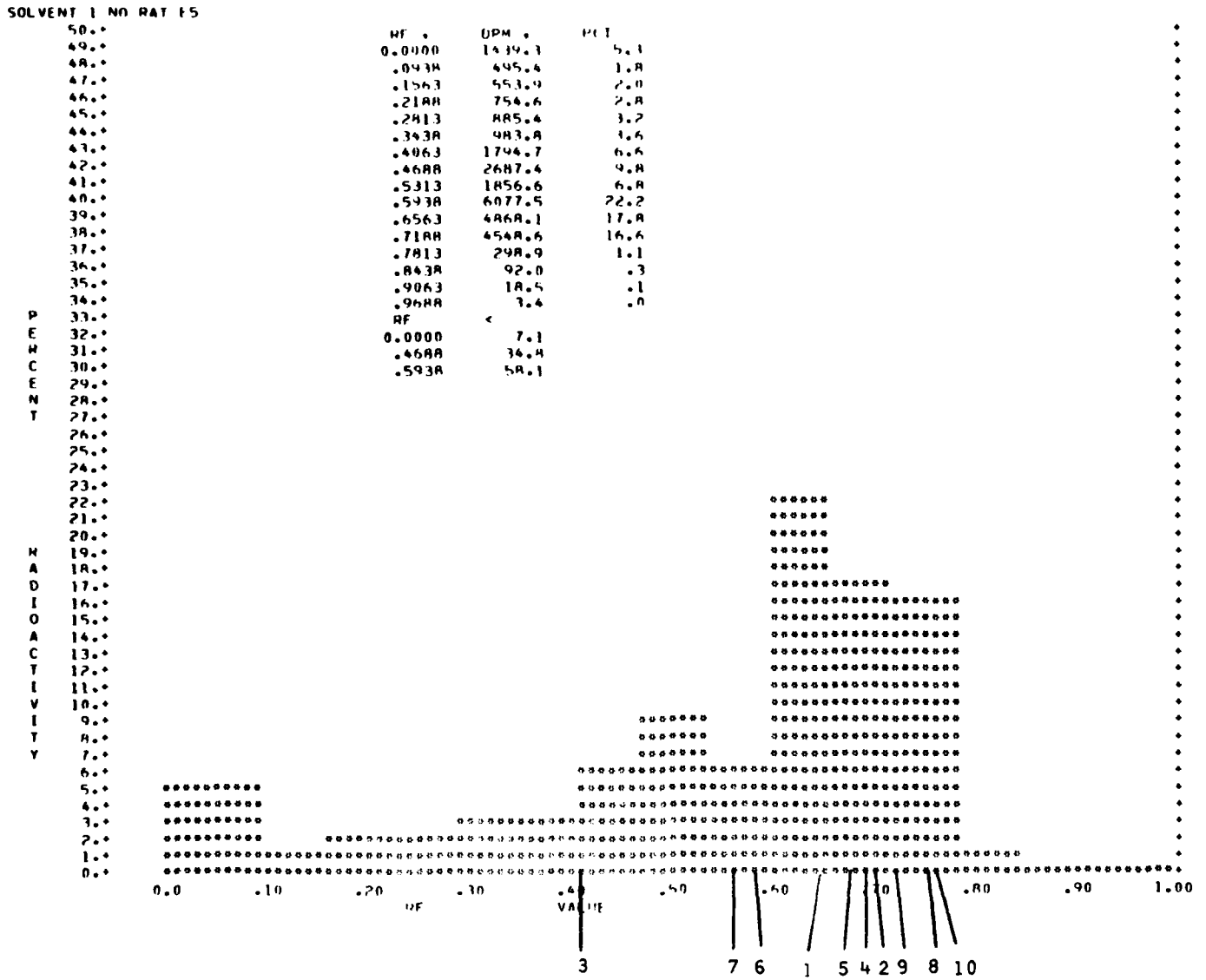


Figure 26-E5: Solvent I

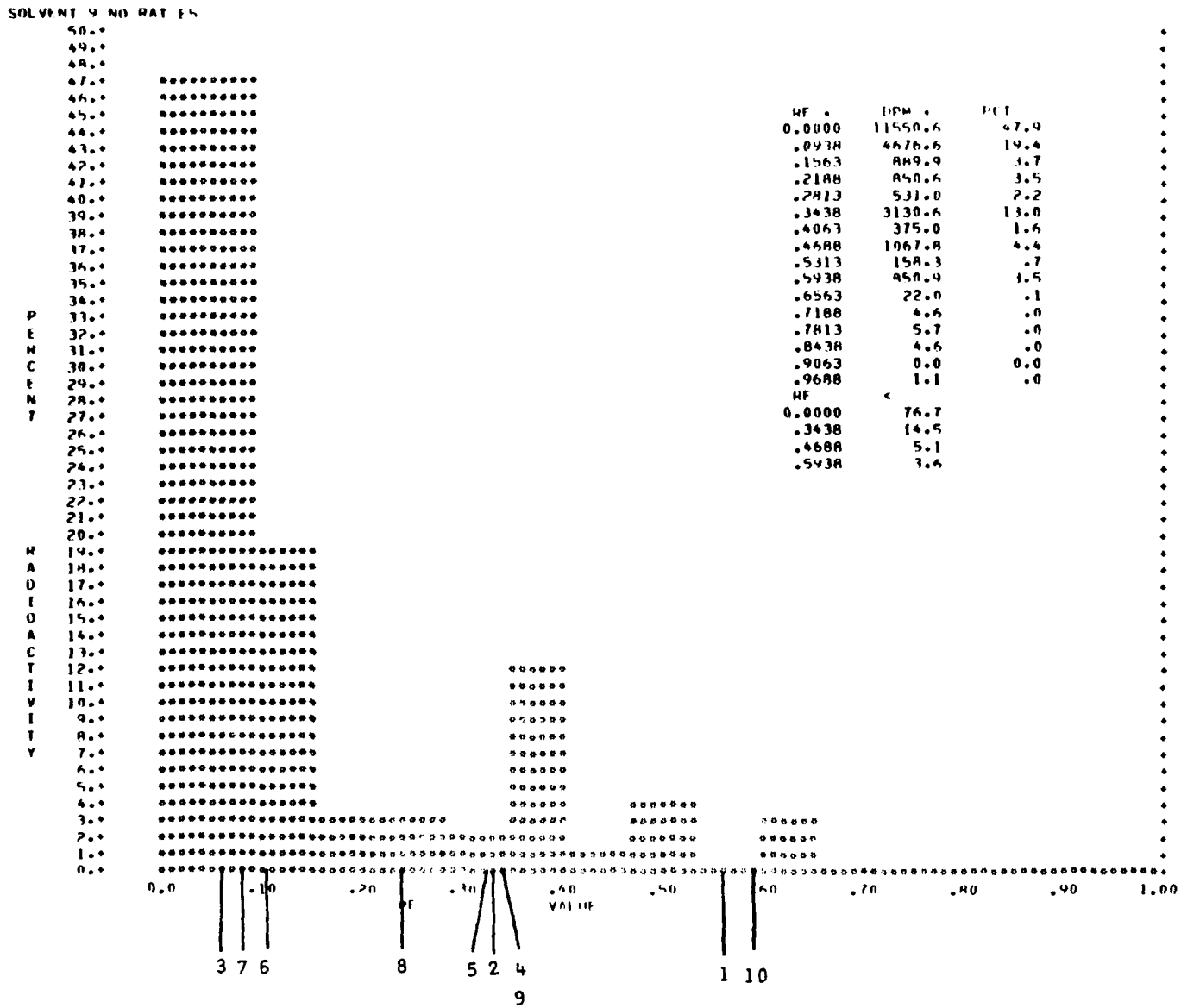


Figure 26-E5: Solvent IX

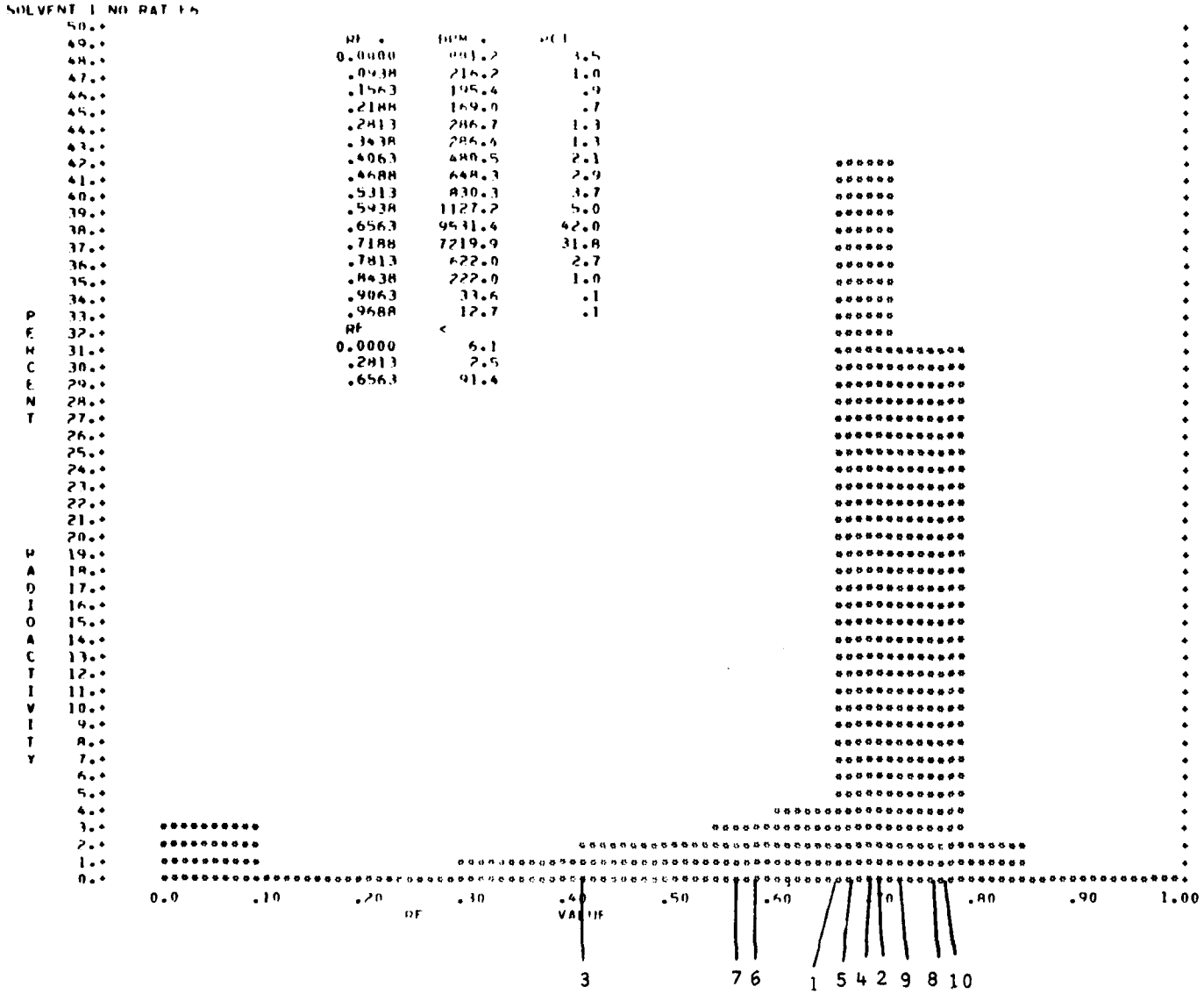


Figure 26-E_6: Solvent I

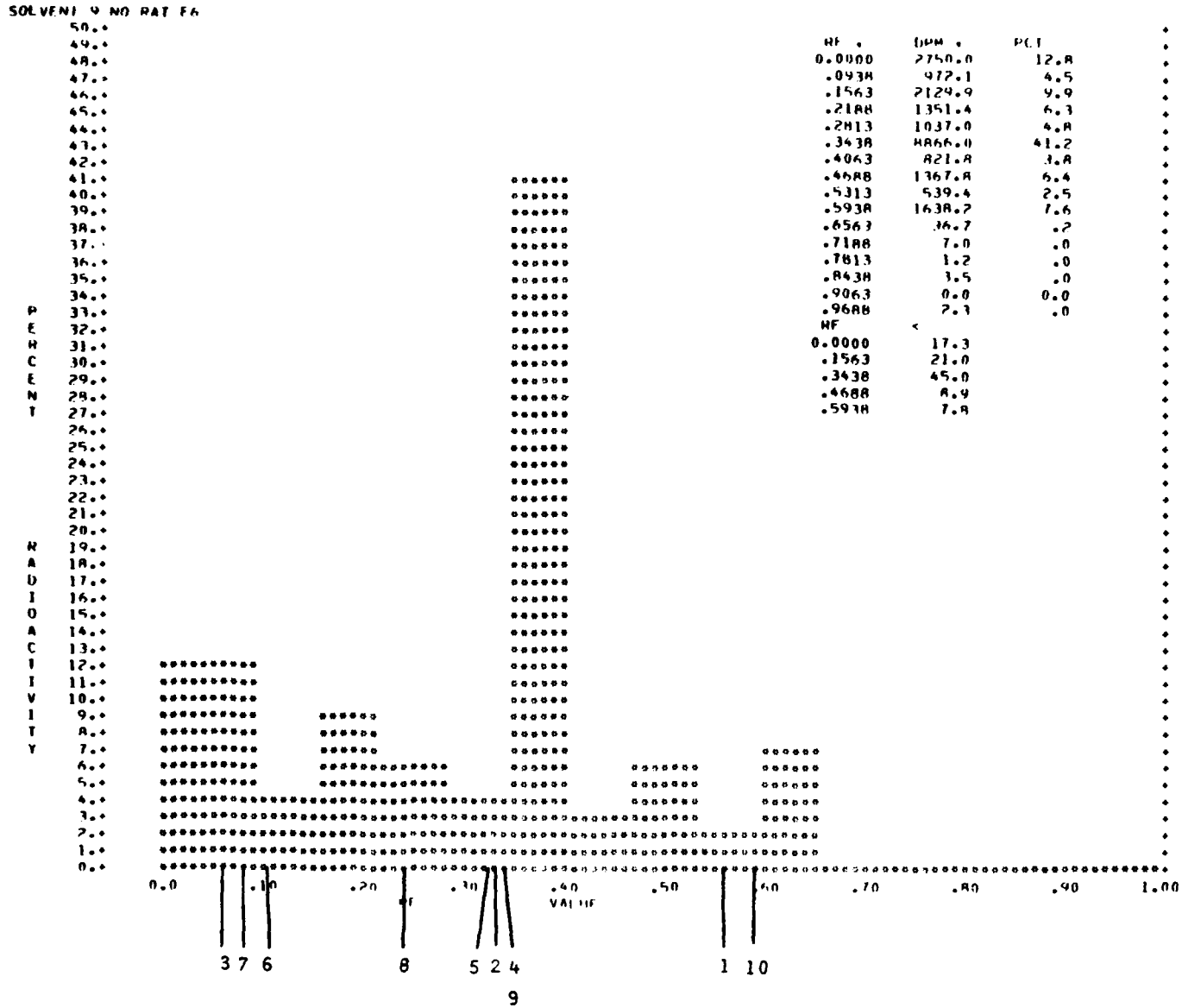


Figure 26-E_6: Solvent IX

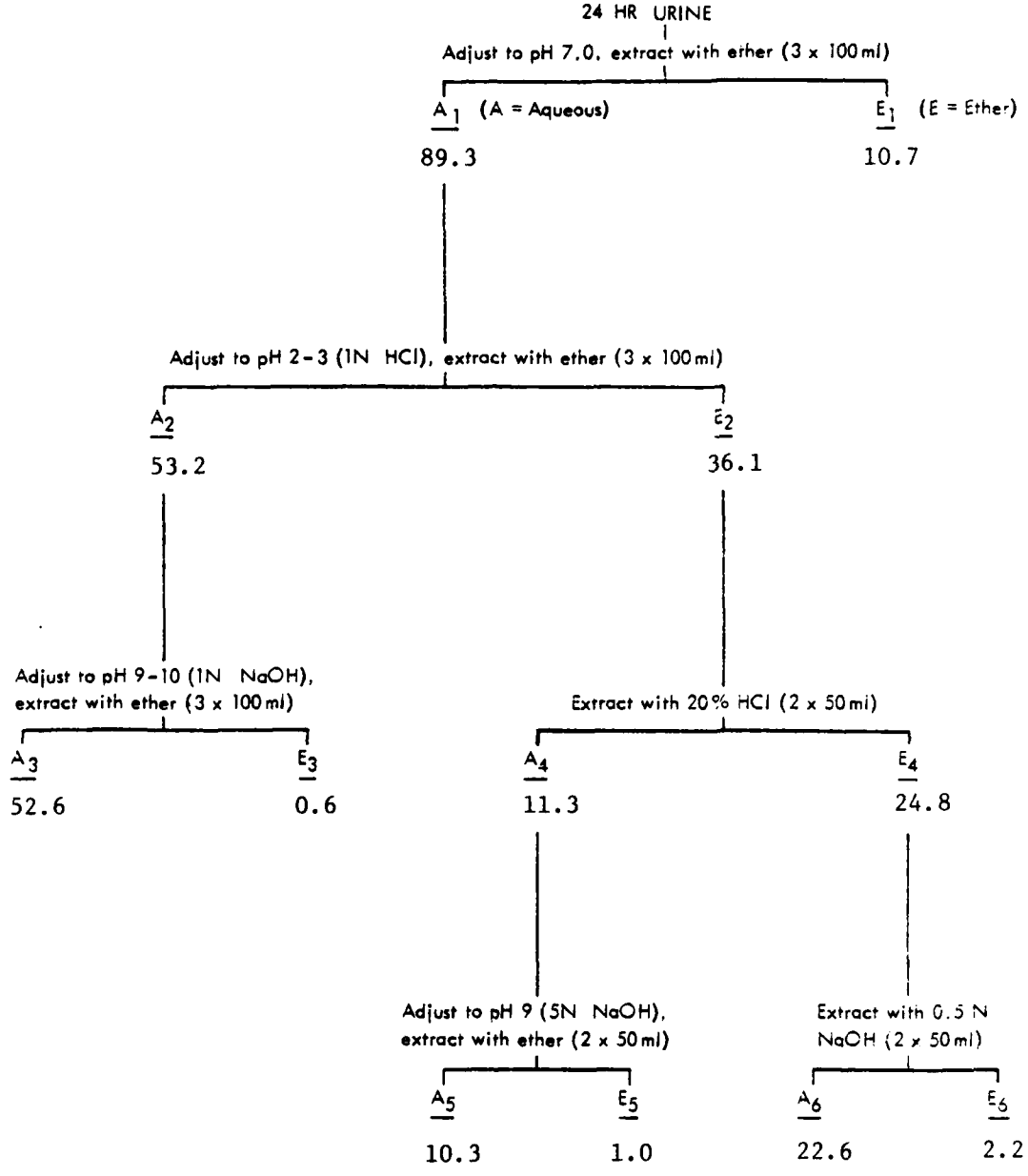


Figure 27: Fractionation of 24-Hr Urine Obtained from Rats Treated Dermally with ^{14}C-TNT. Values indicate the percentage of extractable radioactivity in each fraction.

Figure 28, E_1-E_6: TLC of Ether Products Obtained from 24-Hr Urine of Rats Treated Dermally with ^{14}C-TNT. Extractions were performed at different pH conditions according to the scheme described in the preceeding figure. Solvent I, n-butanol:acetic acid:water, 10:1:1; Solvent IX, toluene:acetic acid, 4:1. Reference metabolites are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzyl alcohol
3. Trinitrobenzoic Acid
4. 4-Amino-2,6-dinitrotoluene
5. 2-Amino-4,6-dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2',6'-Tetranitro-4,4'-azoxytoluene

Figure 28 follows

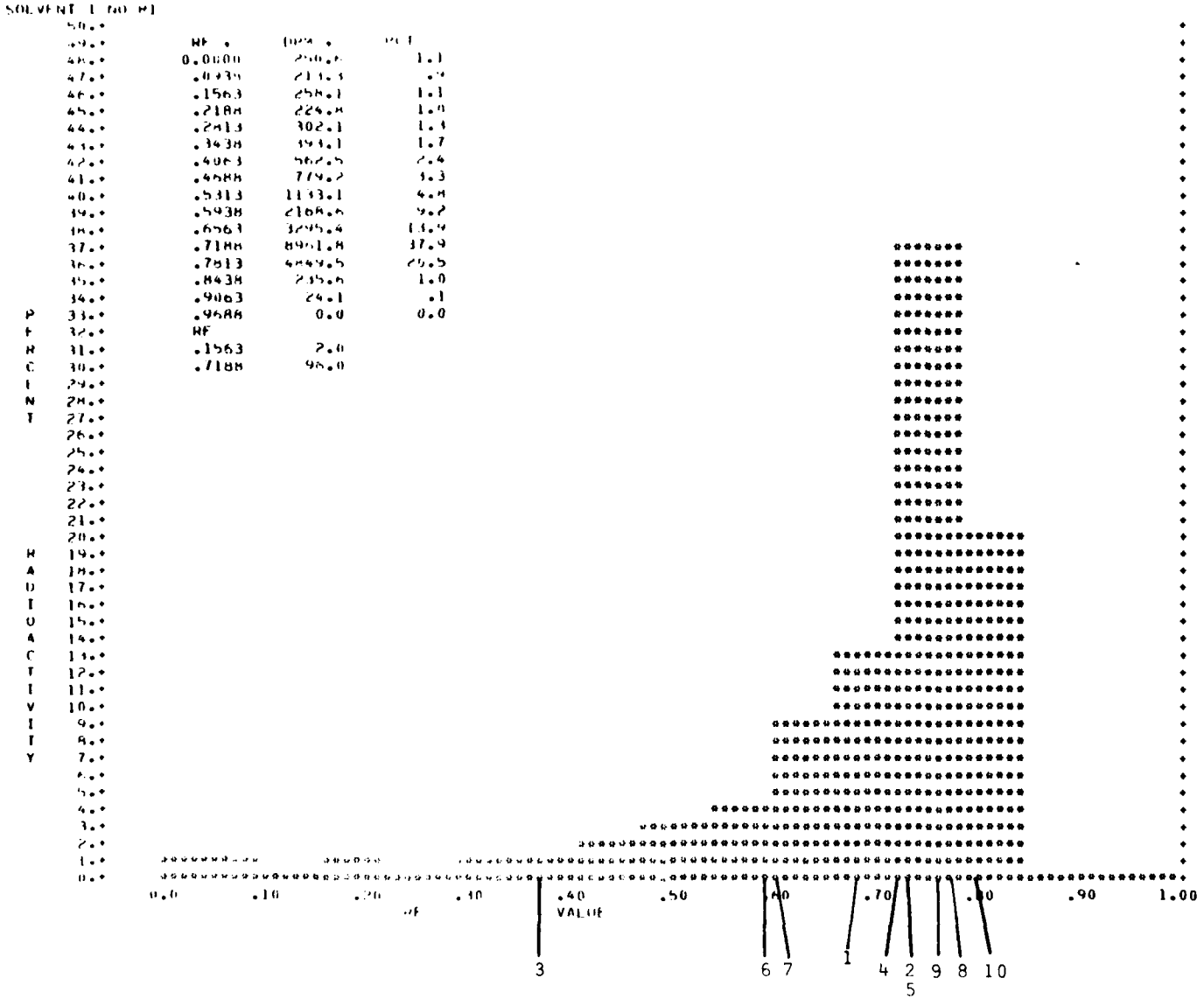


Figure 28-E_1: Solvent I.

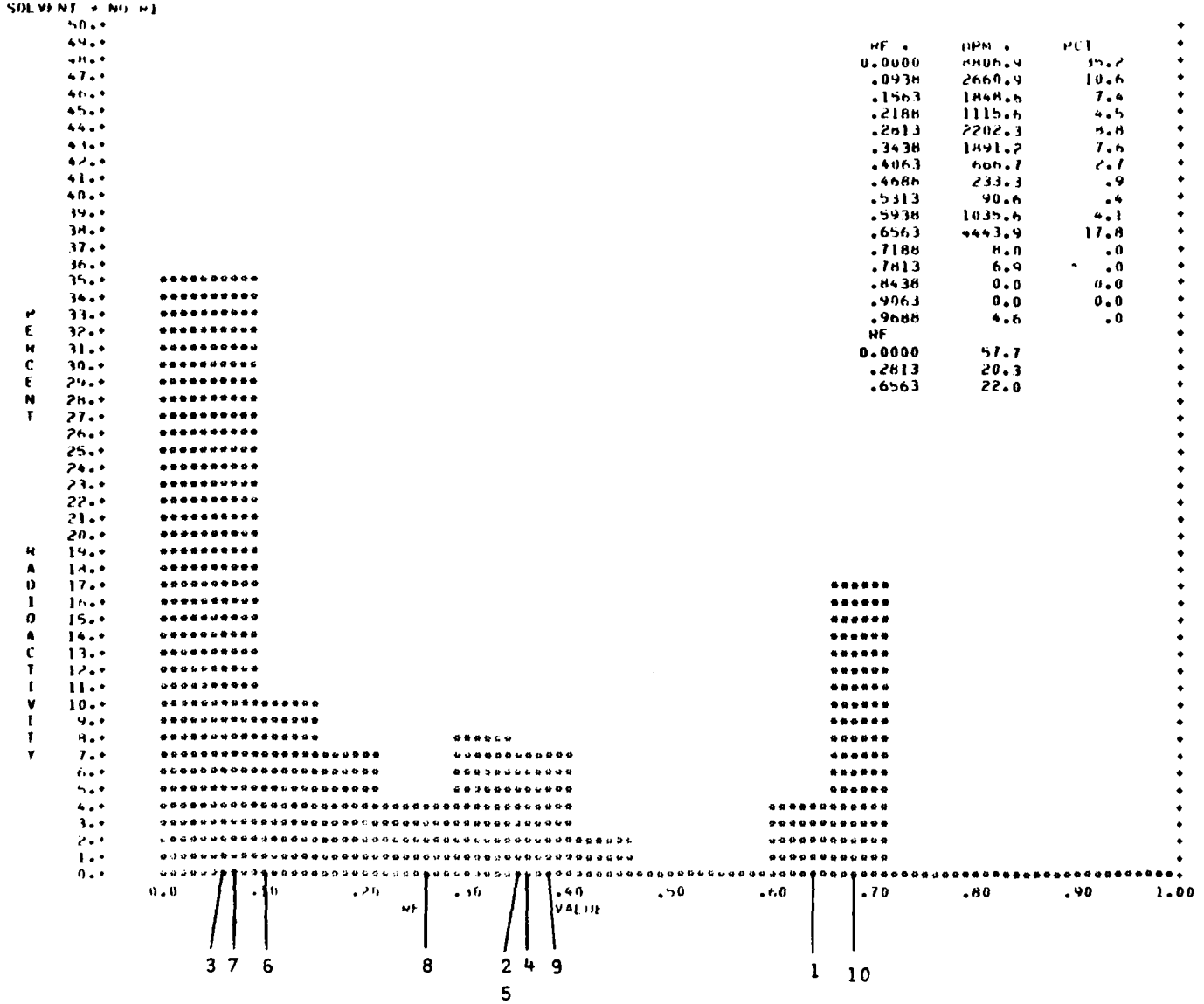


Figure 28-E_1: Solvent IX.

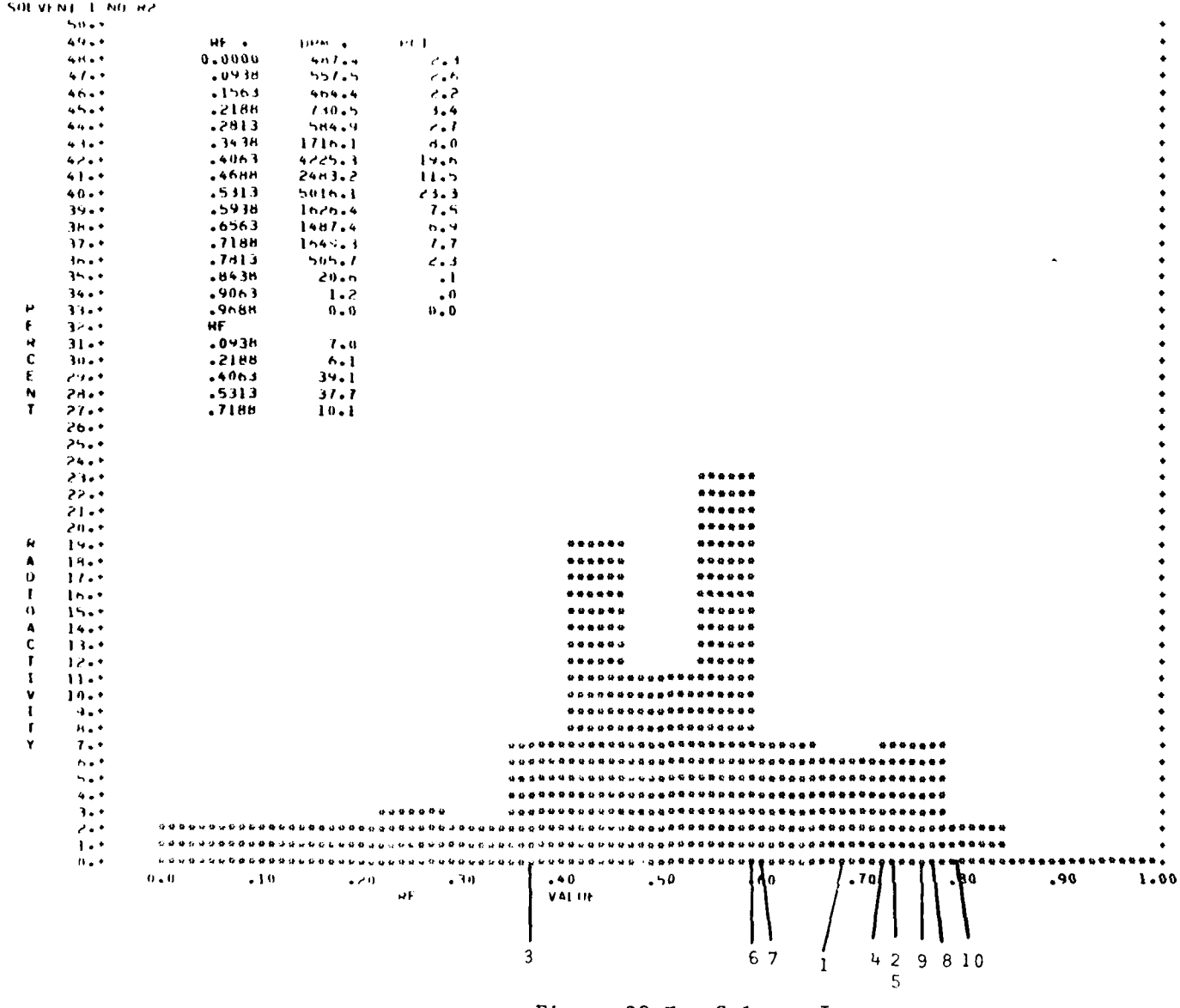


Figure 28-E_2: Solvent I.

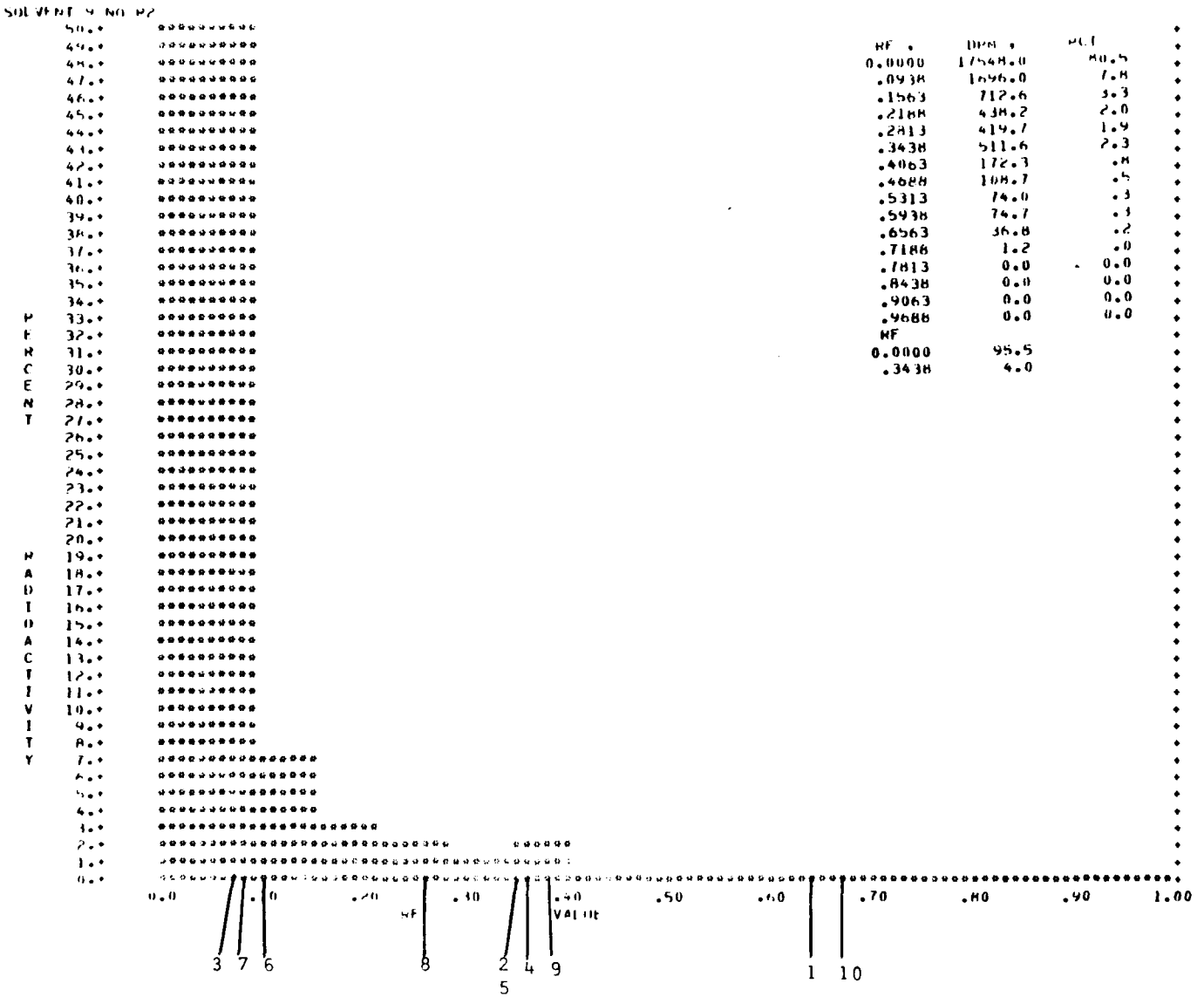


Figure 28-E_2: Solvent IX.

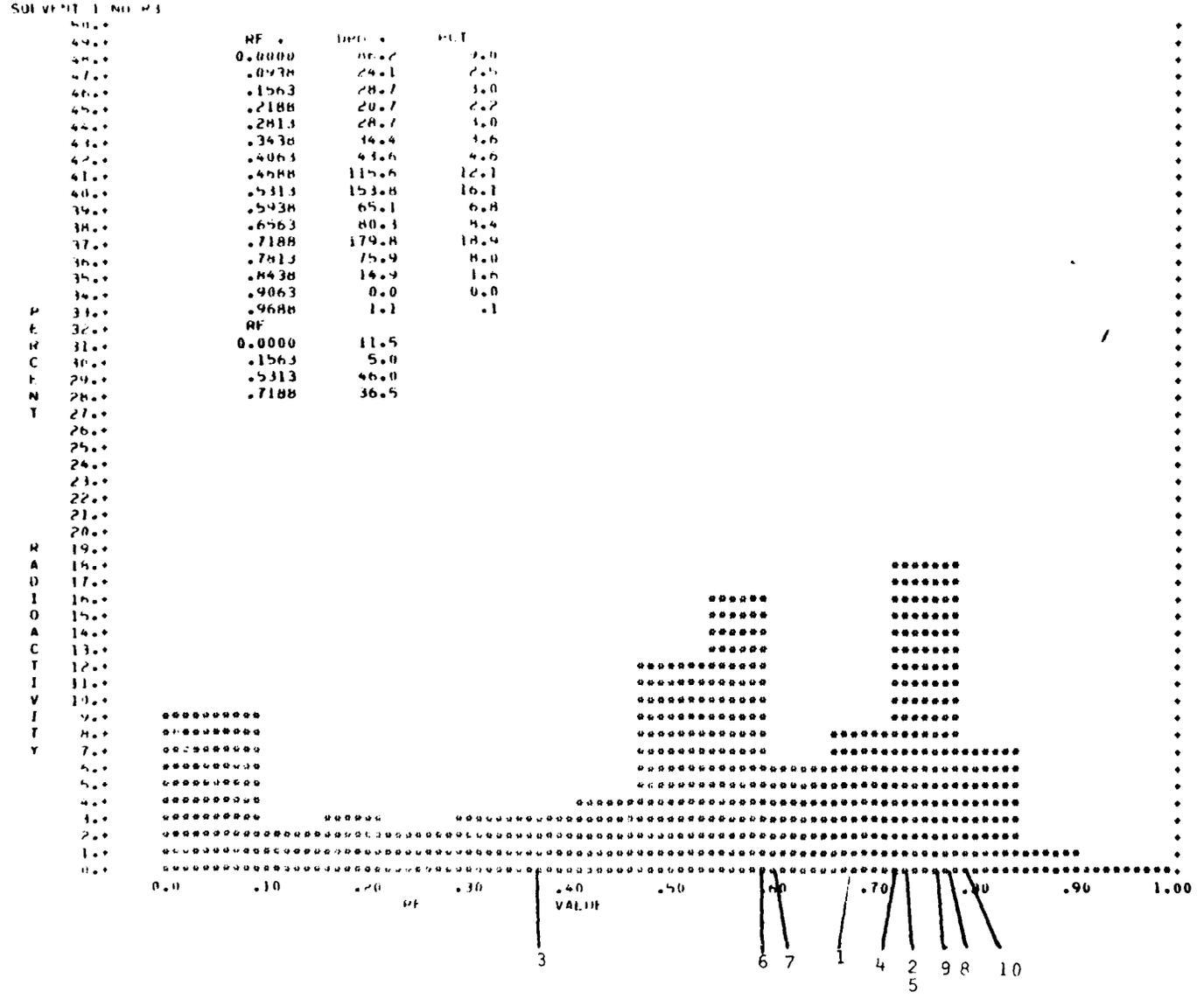


Figure 28-E3: Solvent I.

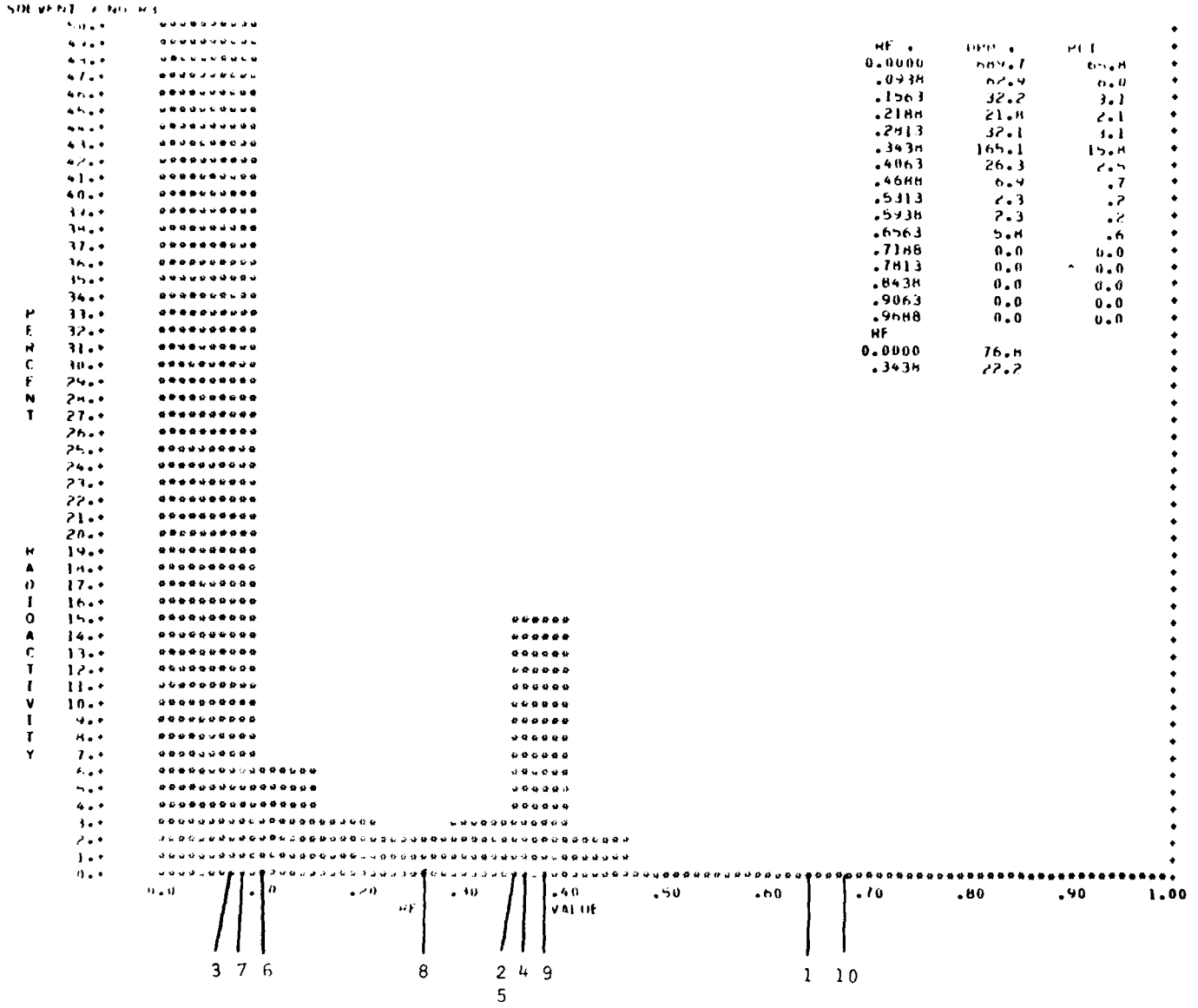


Figure 28-E_3: Solvent IX.

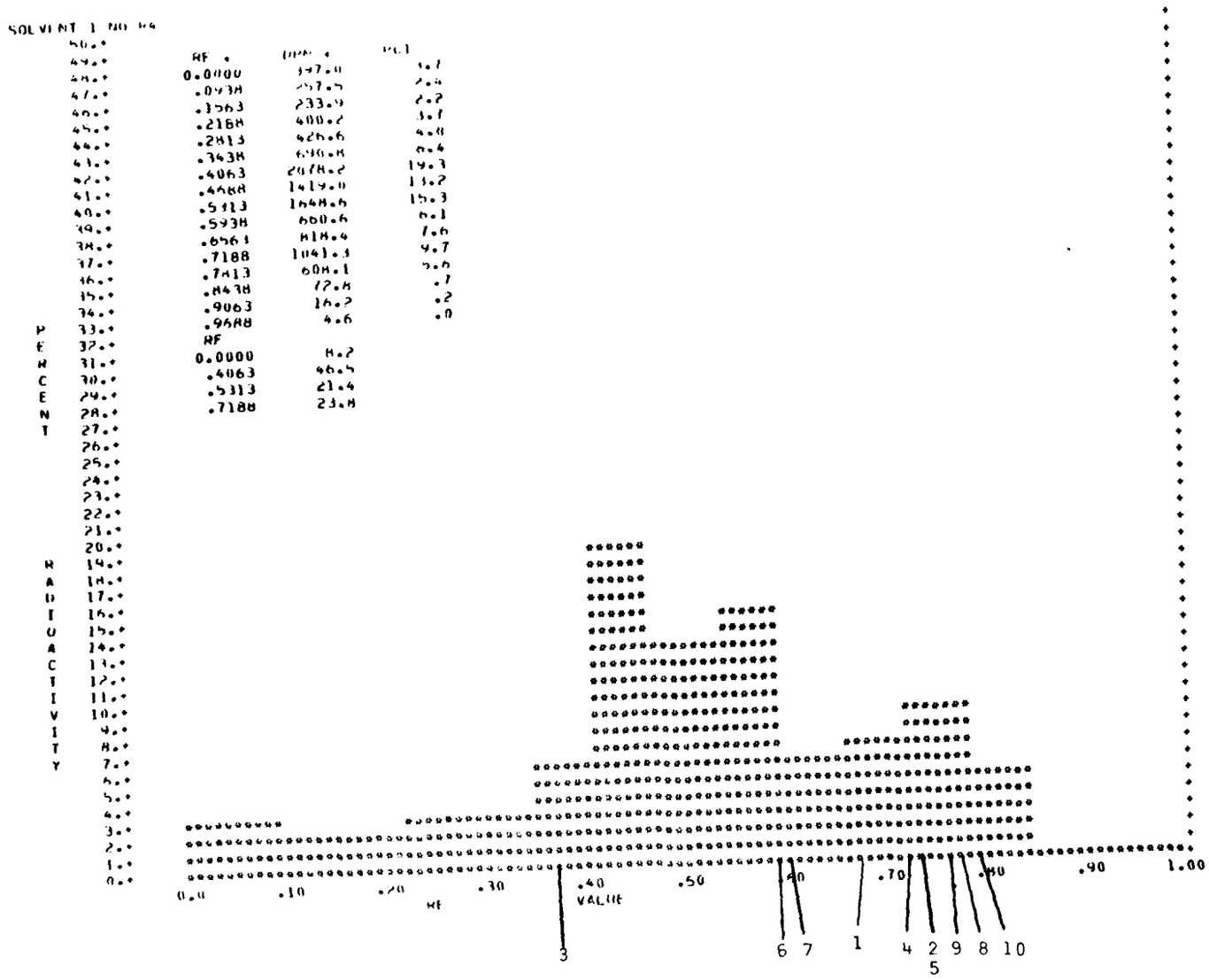


Figure 28-E_4: Solvent I.

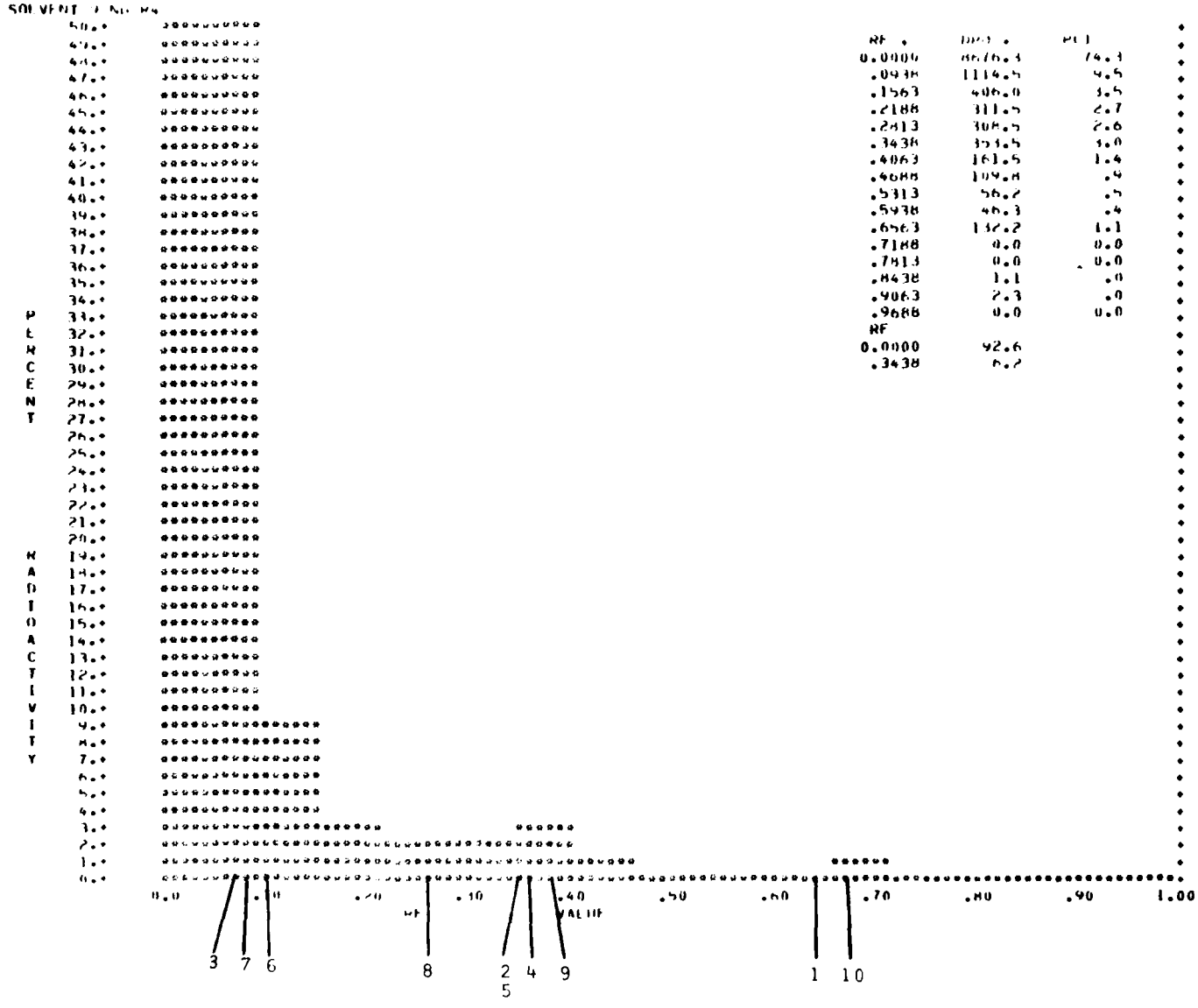


Figure 28-E_4: Solvent IX.

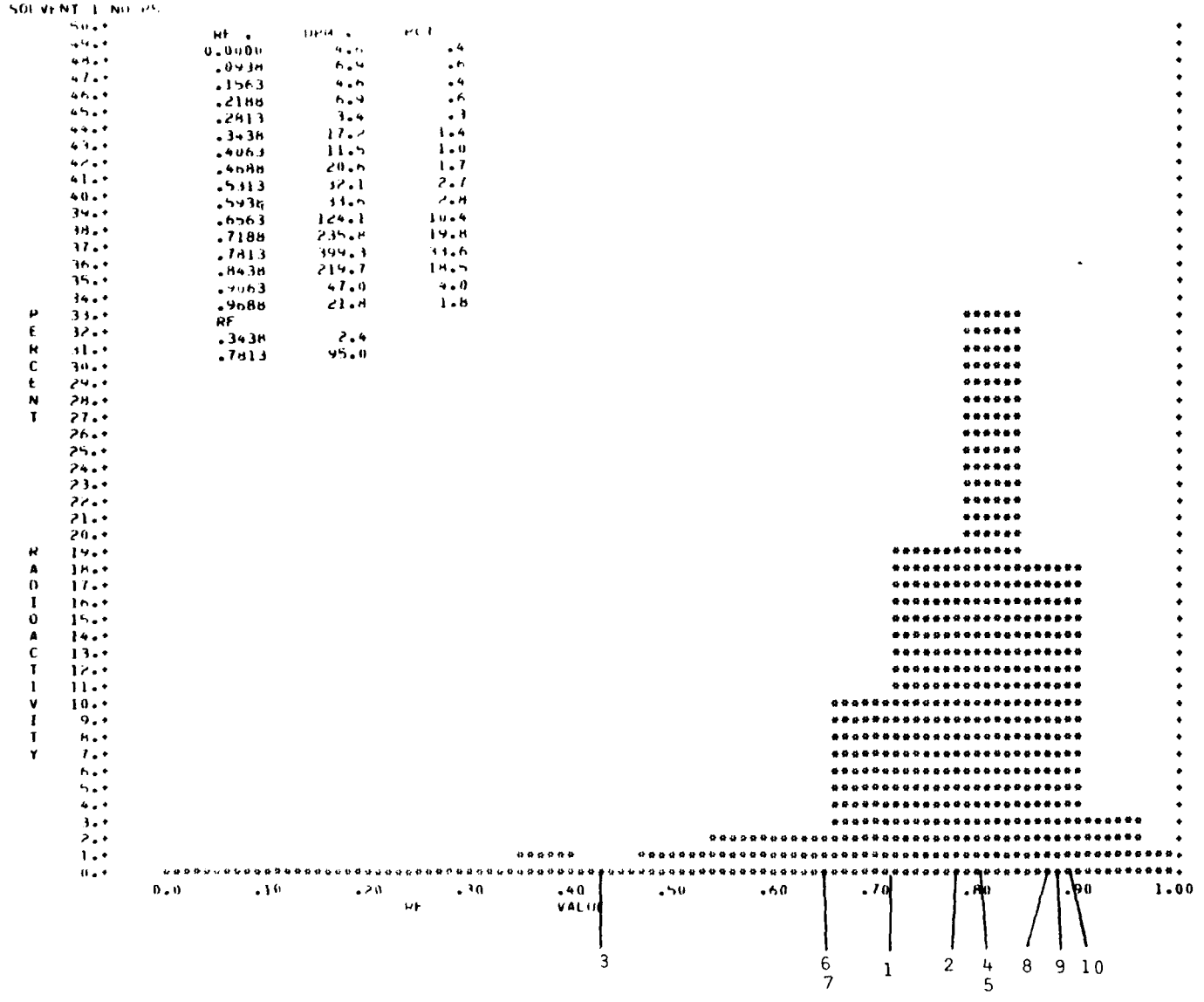


Figure 28-E_5: Solvent I.

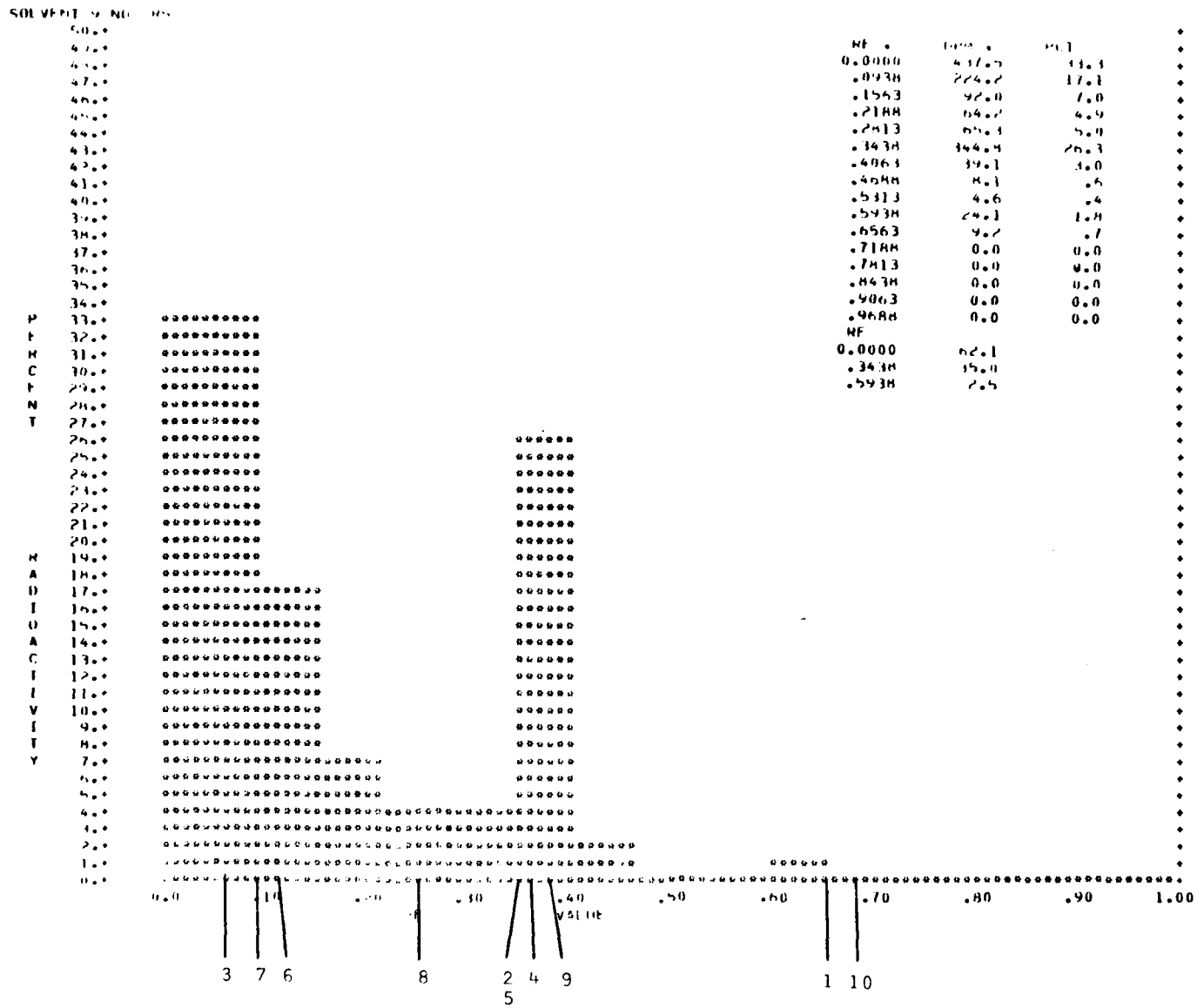


Figure 28-E_5: Solvent IX.

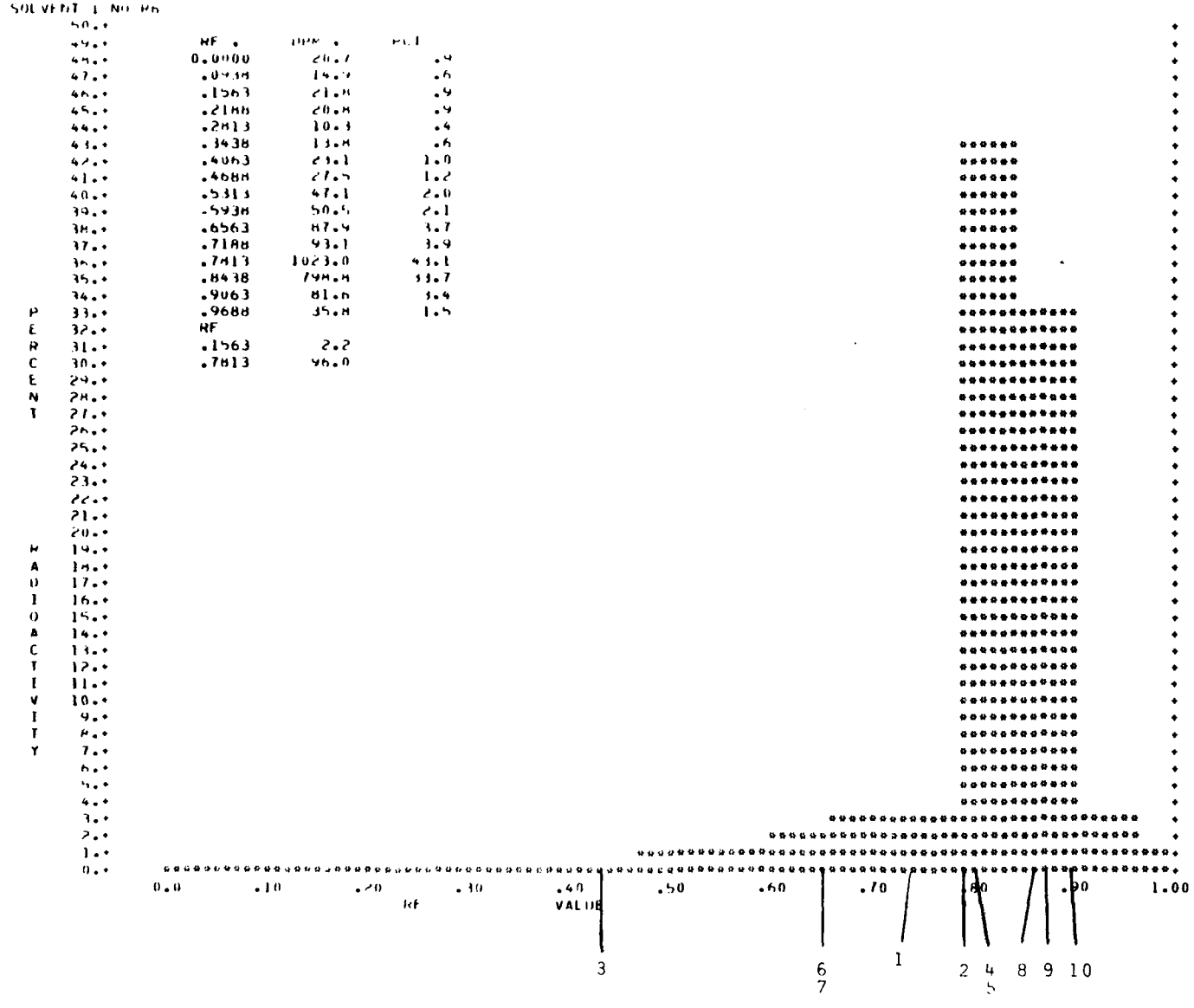


Figure 28-E_6: Solvent I.

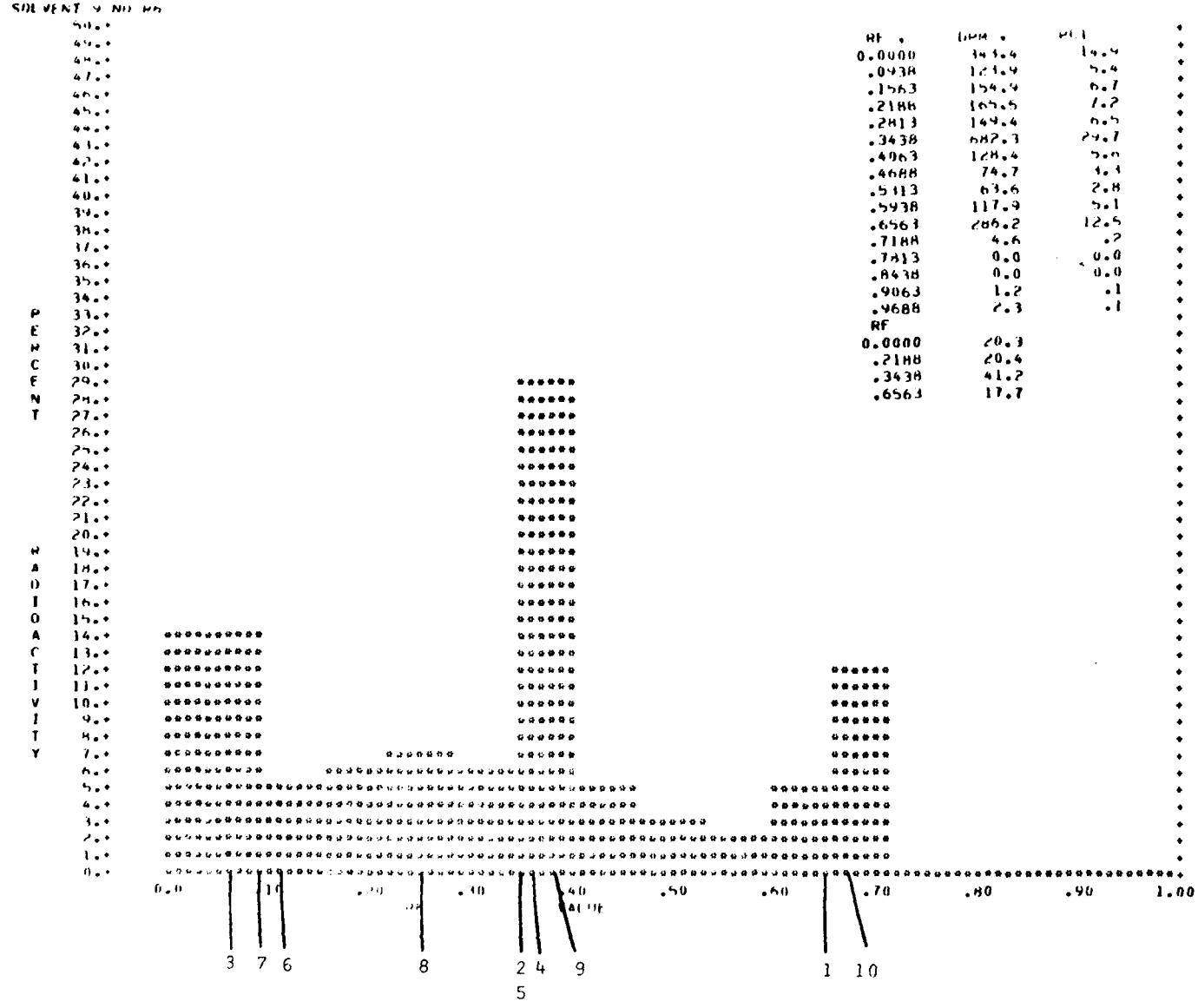


Figure 28-E_6: Solvent IX.

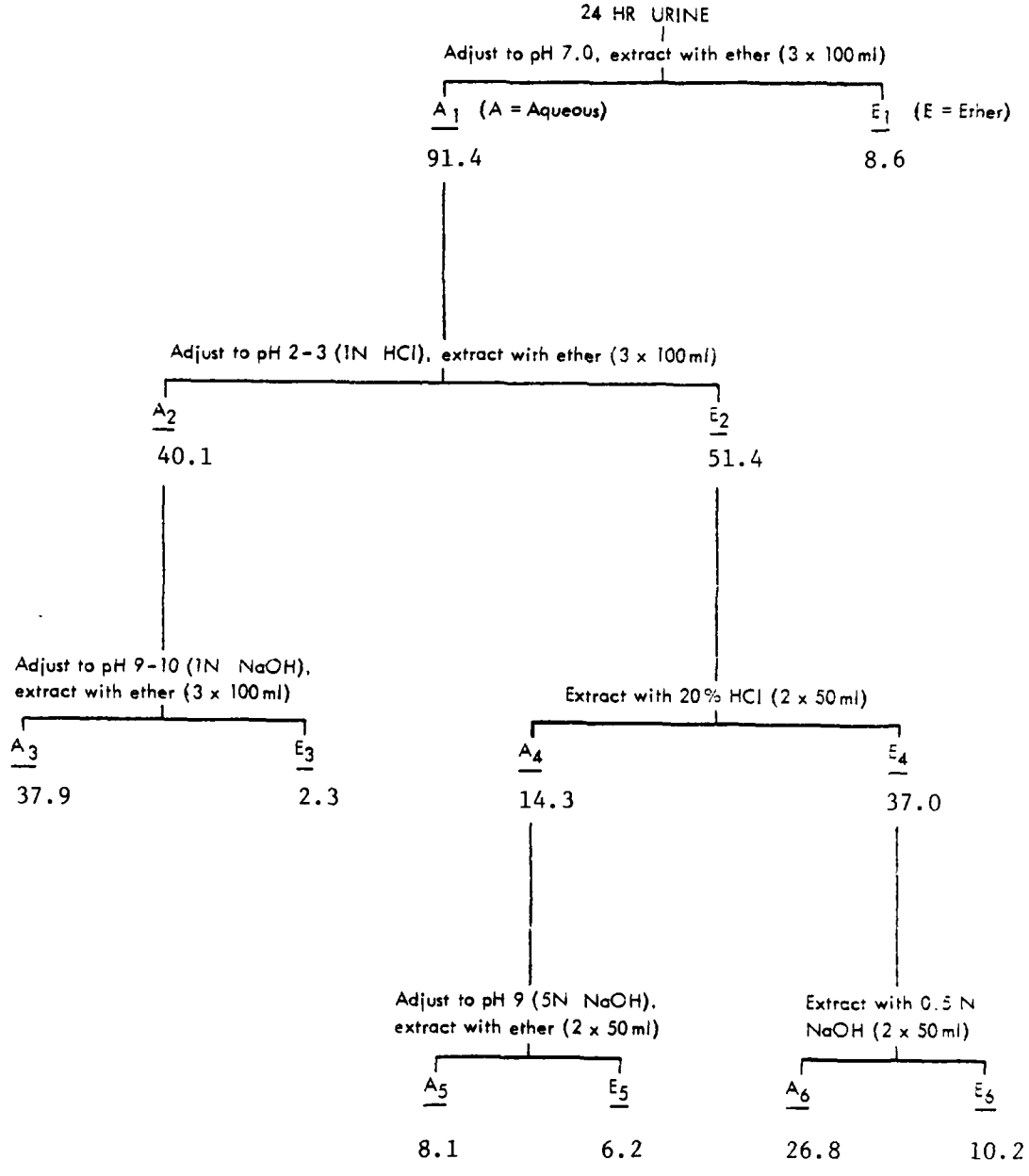


Figure 29: Fractionation of 24-Hr Urine Obtained from Mice Treated Orally with ^{14}C-TNT. Values indicate the percentage of extractable radioactivity in each fraction.

Figure 30, E_1-E_6: TLC of Ether-Extractable Product Obtained from 24-Hr Urine of Mice Treated Orally with ^{14}C-TNT. Extractions were performed at different pH conditions according to the scheme described in the preceding figure. Solvent I, n-butanol:acetic acid:water, 10:1:1; Solvent IX, toluene:acetic acid, 4:1. For reference metabolites (1-10) see Figure 26 or Table 19. (E_5 fraction was spilled.) Reference metabolites are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzyl alcohol
3. Trinitrobenzoic Acid
4. 4-Amino-2,6-dinitrotoluene
5. 2-Amino-4,6-dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2',6'-Tetranitro-4,4'-azoxytoluene

Figure 30 follows

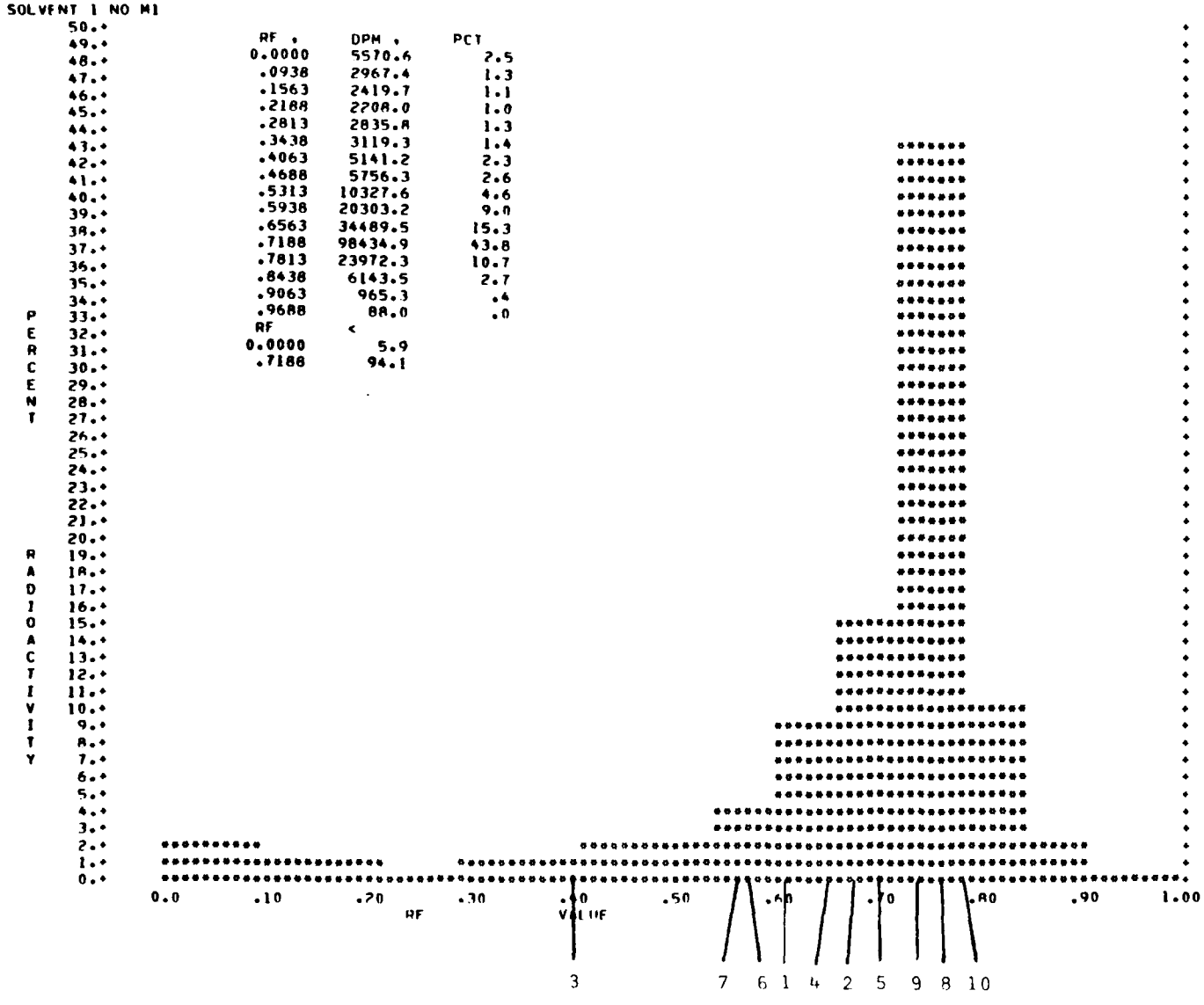


Figure 30-E_1: Solvent I

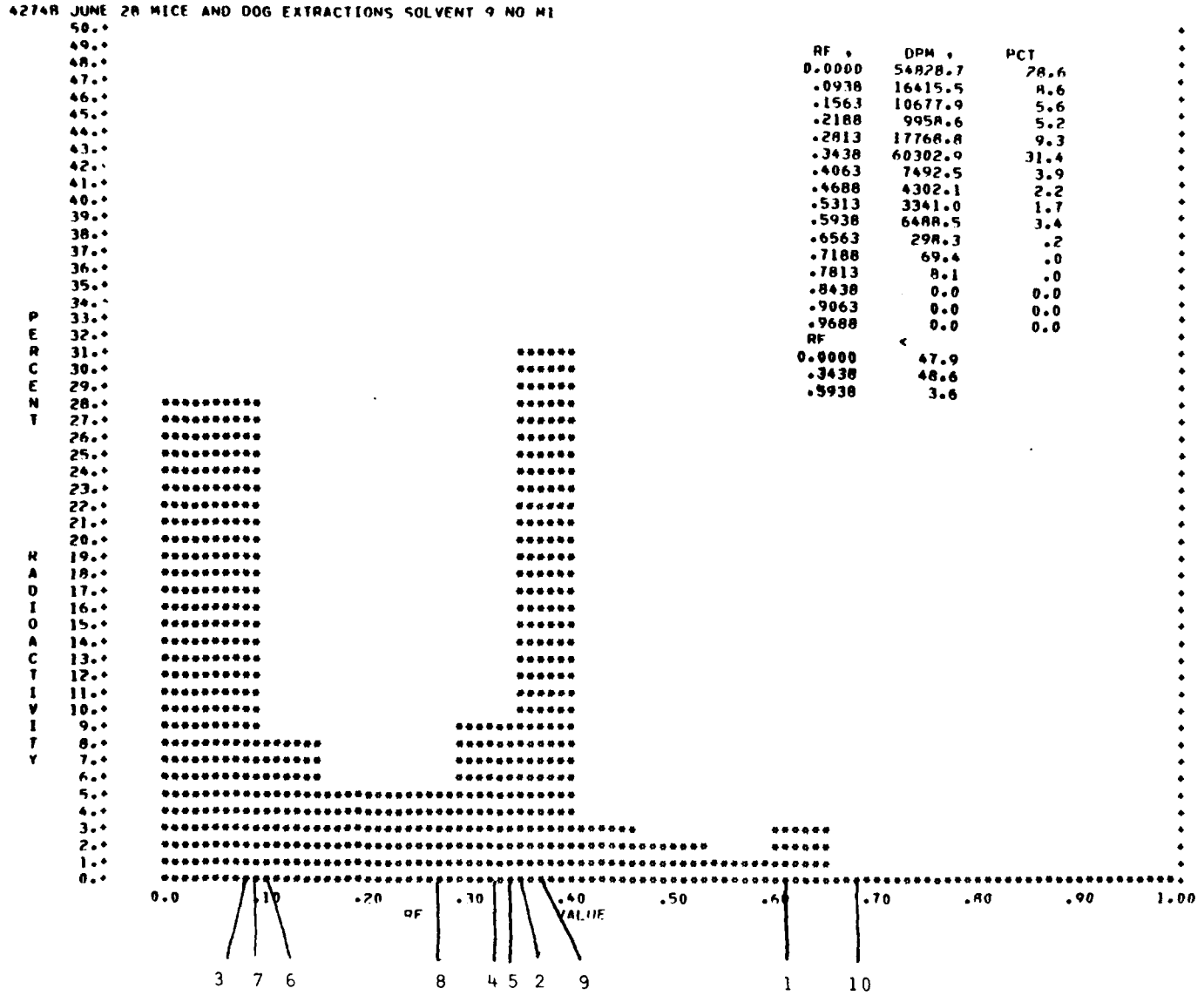


Figure 30-E_1: Solvent IX

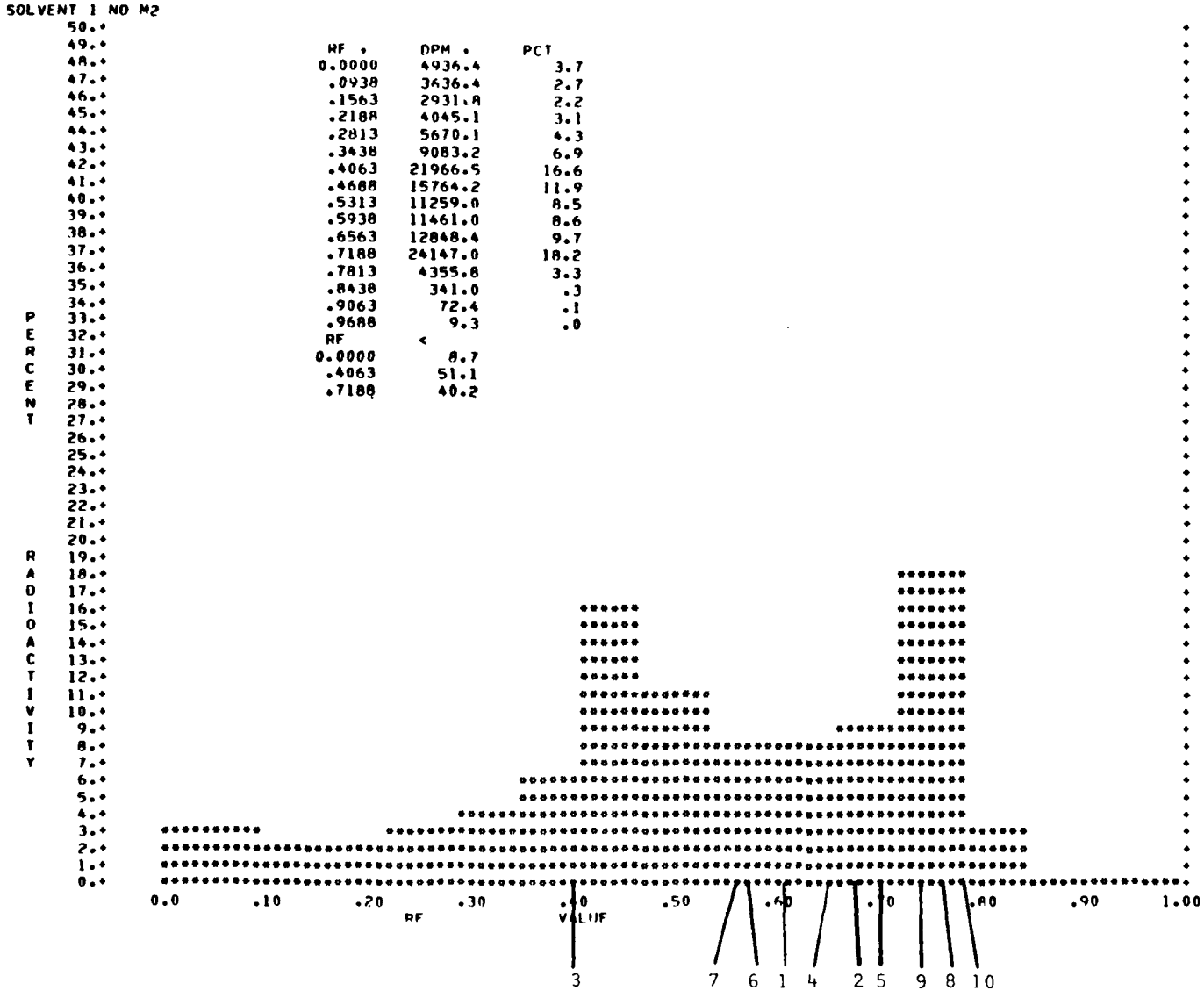
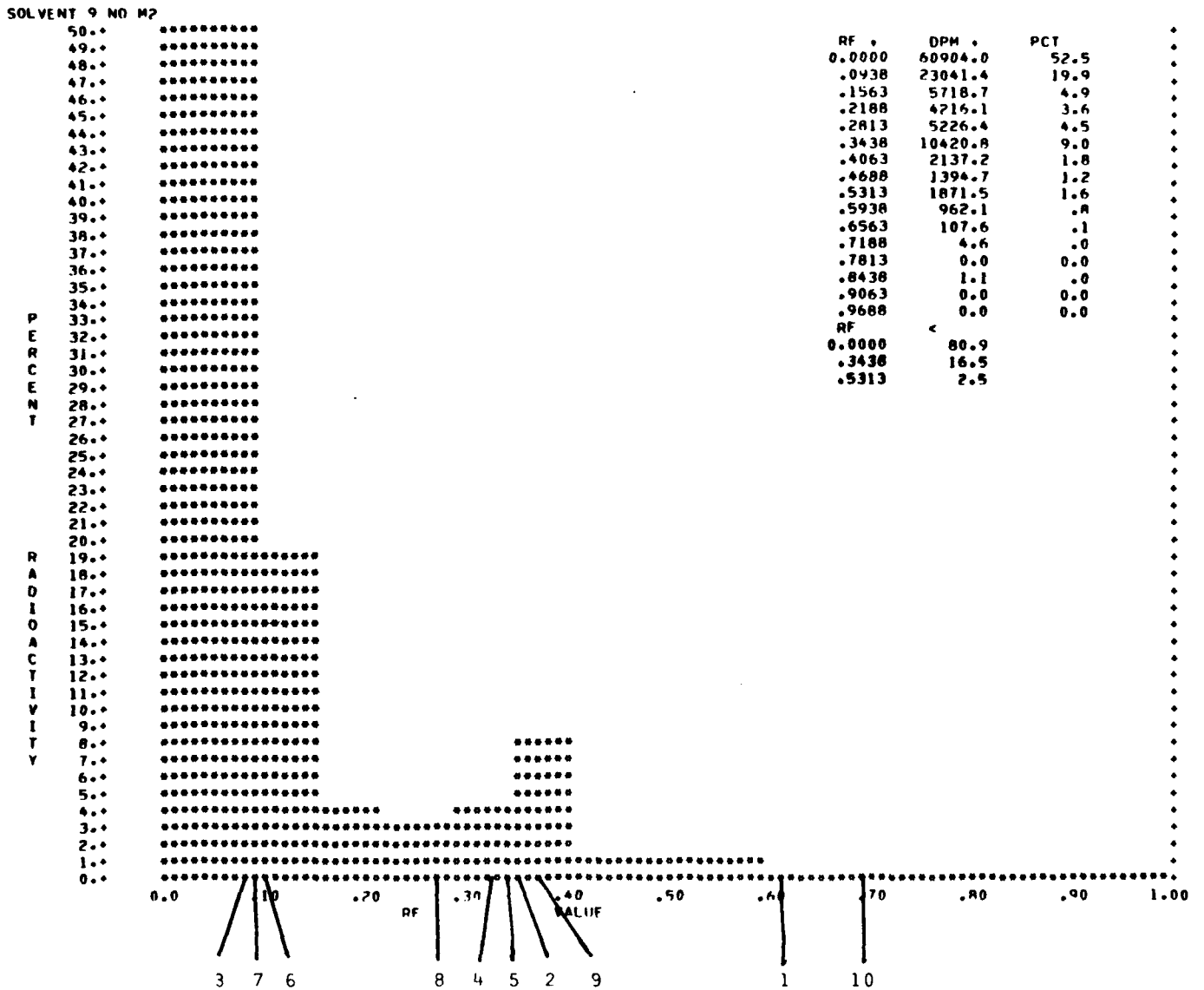


Figure 30-E_2: Solvent I



RF	DPM	PCT
0.0000	60904.0	52.5
.0938	23041.4	19.9
.1563	5718.7	4.9
.2188	4216.1	3.6
.2813	5226.4	4.5
.3438	10420.8	9.0
.4063	2137.2	1.8
.4688	1394.7	1.2
.5313	1871.5	1.6
.5938	962.1	.8
.6563	107.6	.1
.7188	4.6	.0
.7813	0.0	0.0
.8438	1.1	.0
.9063	0.0	0.0
.9688	0.0	0.0

RF	<
0.0000	80.9
.3438	16.5
.5313	2.5

Figure 30-E_2: Solvent IX

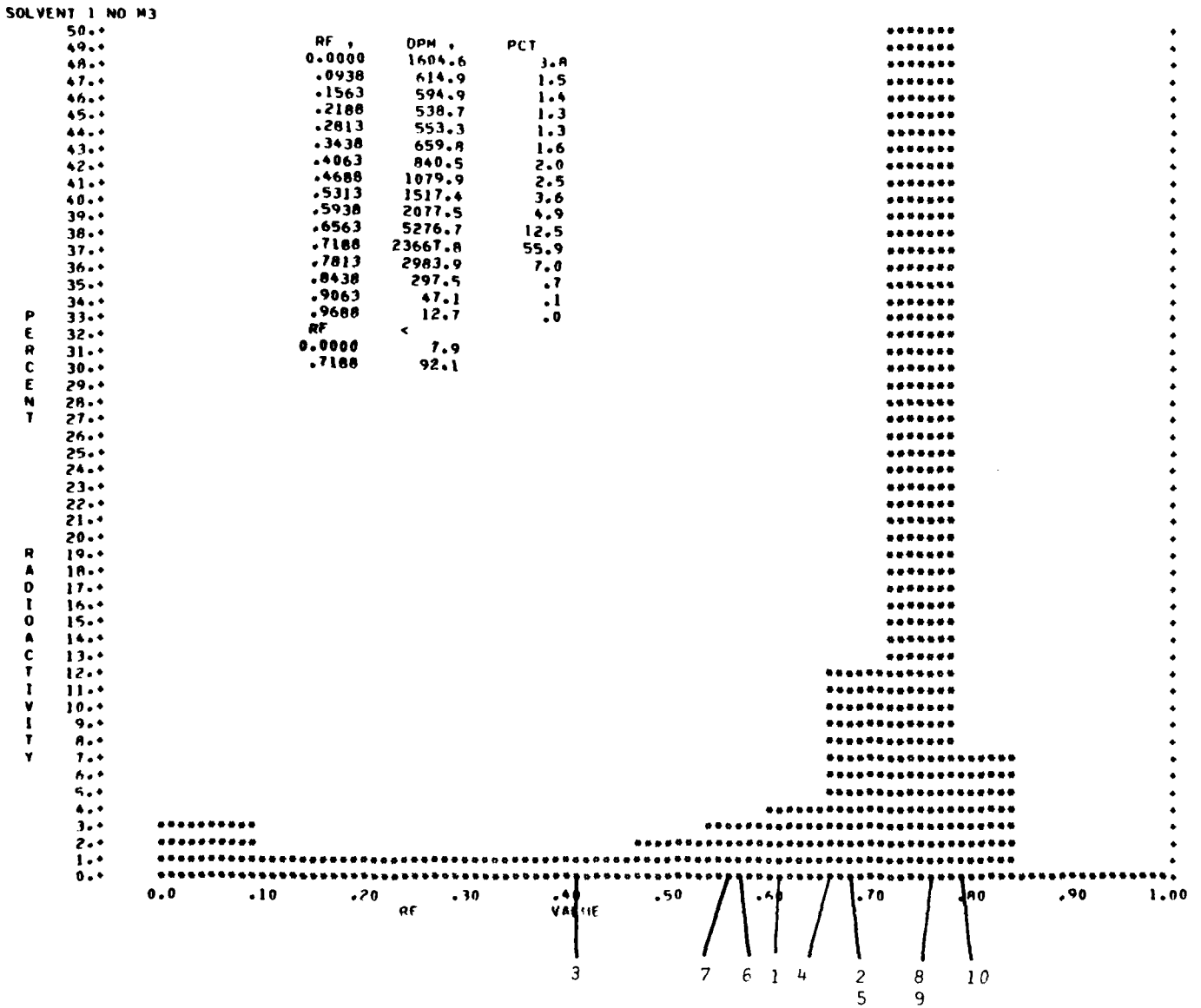


Figure 30-E_3: Solvent I

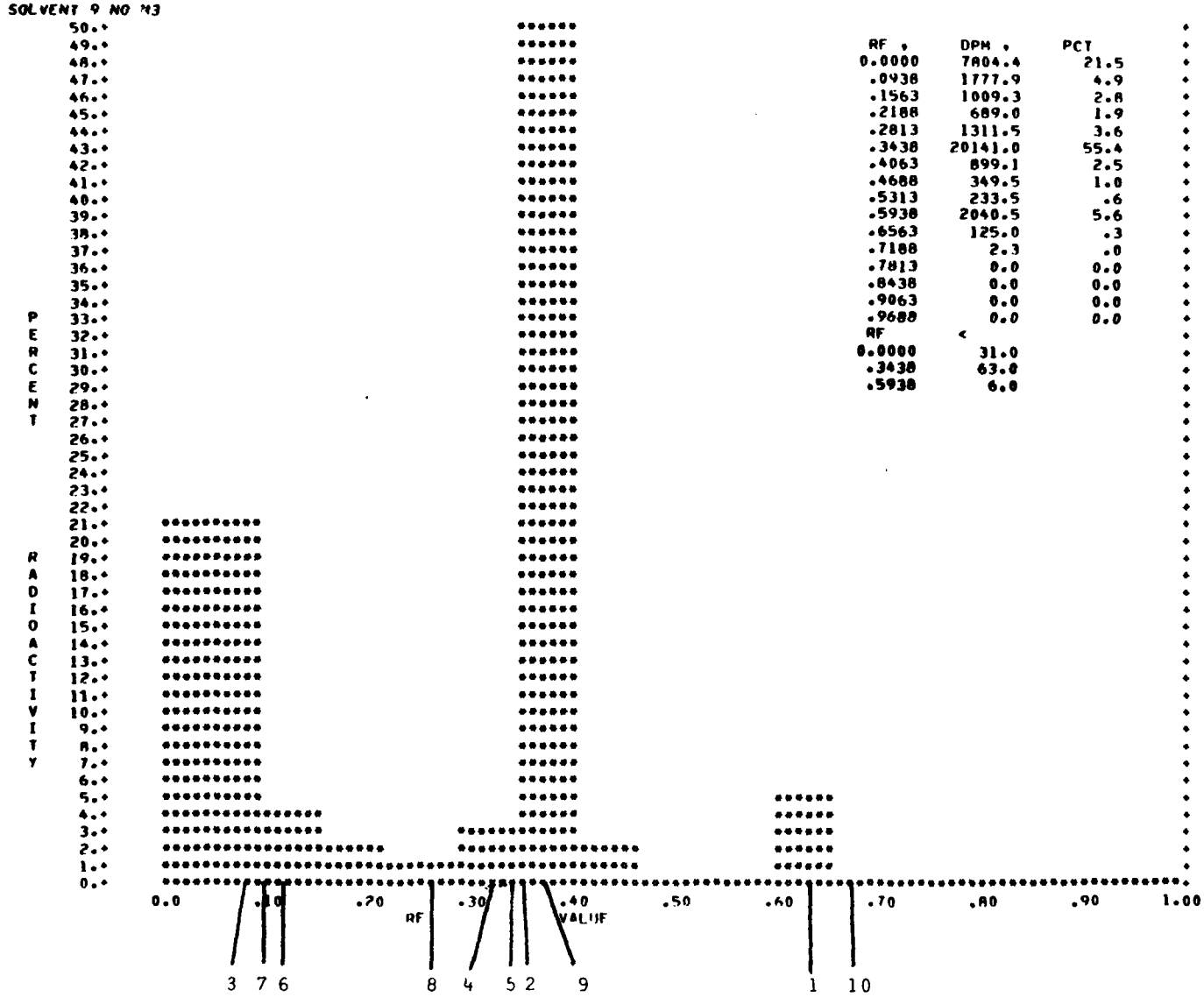


Figure 30-E_3: Solvent IX

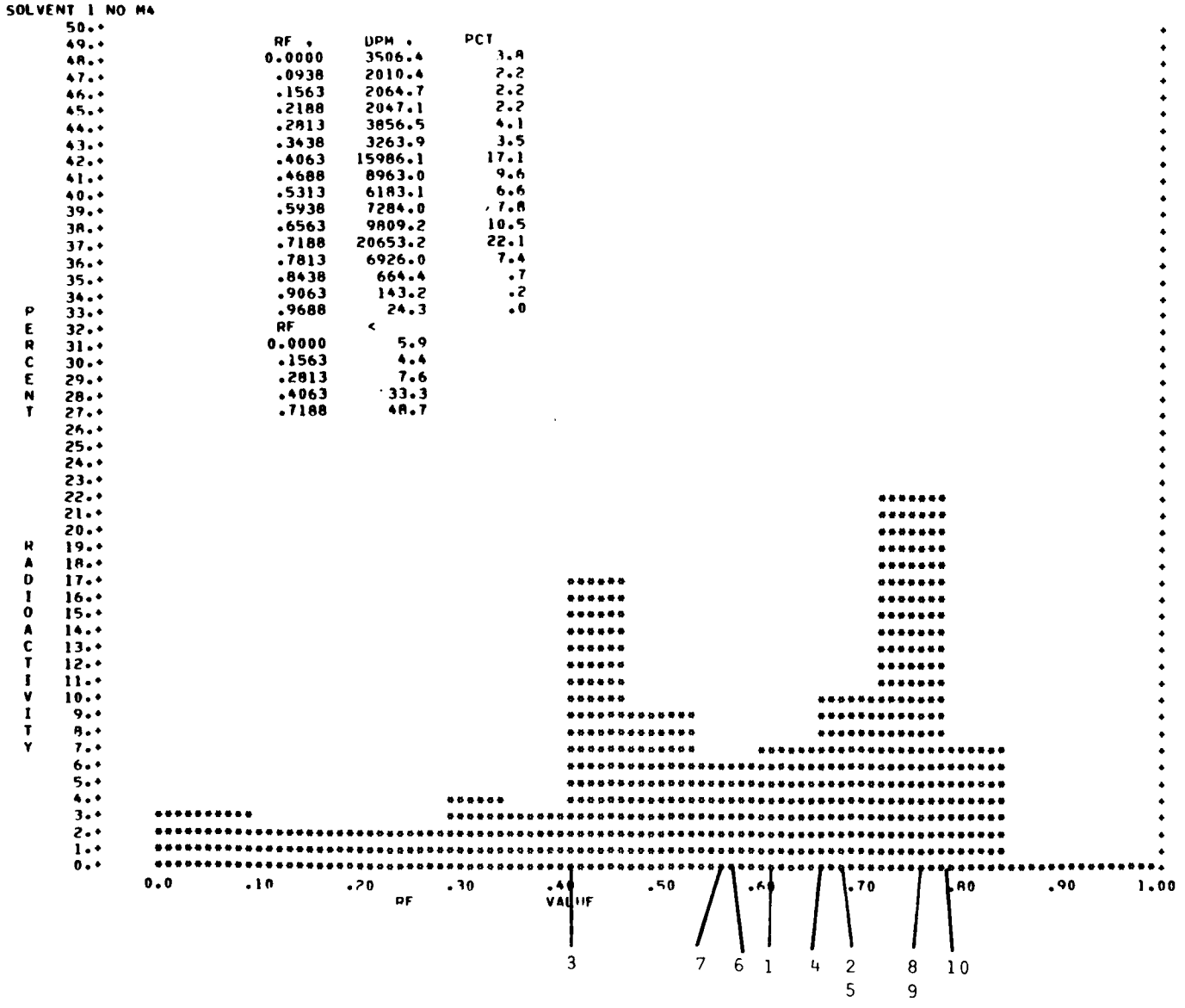


Figure 30-E4: Solvent I

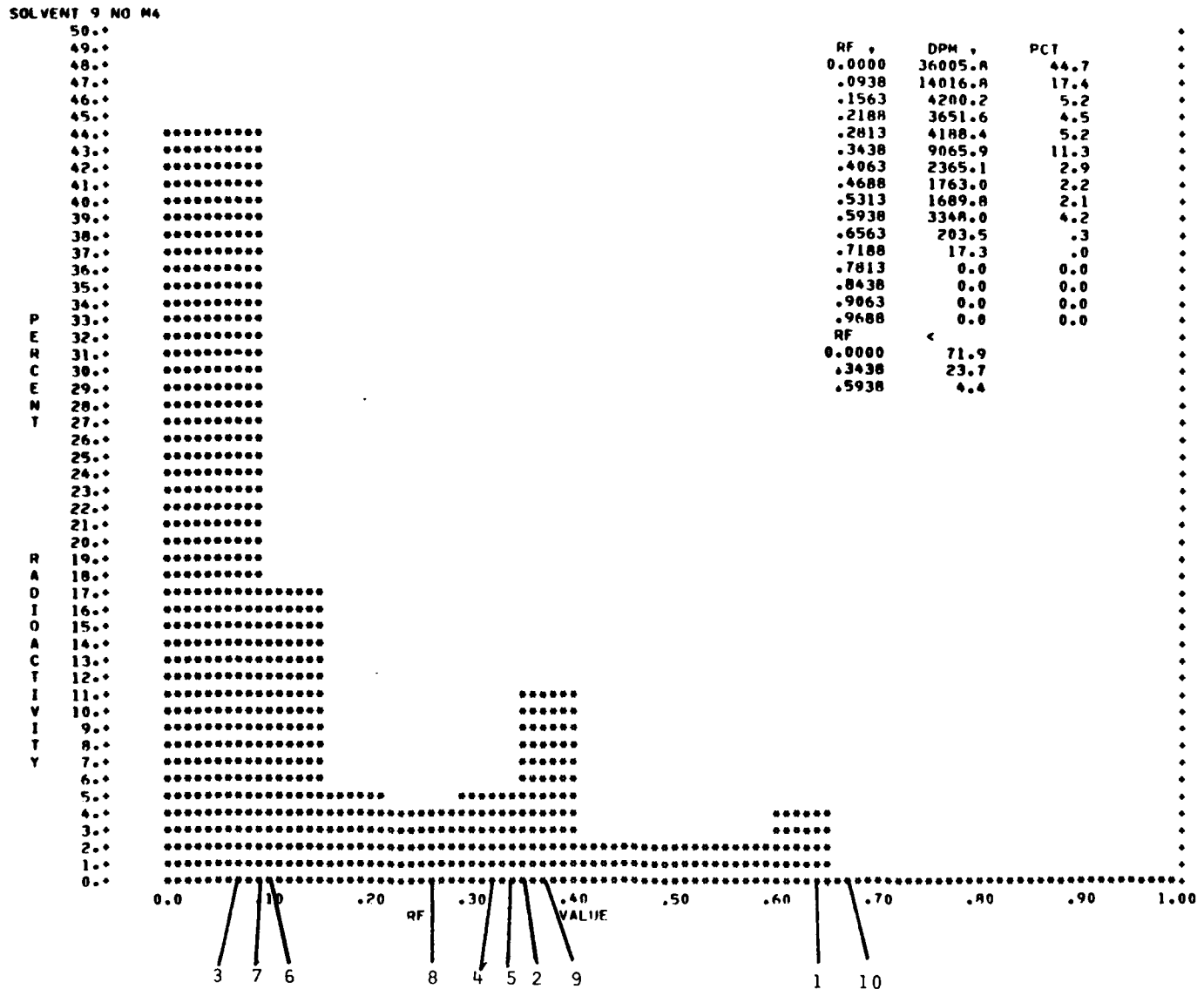


Figure 30-E4: Solvent IX

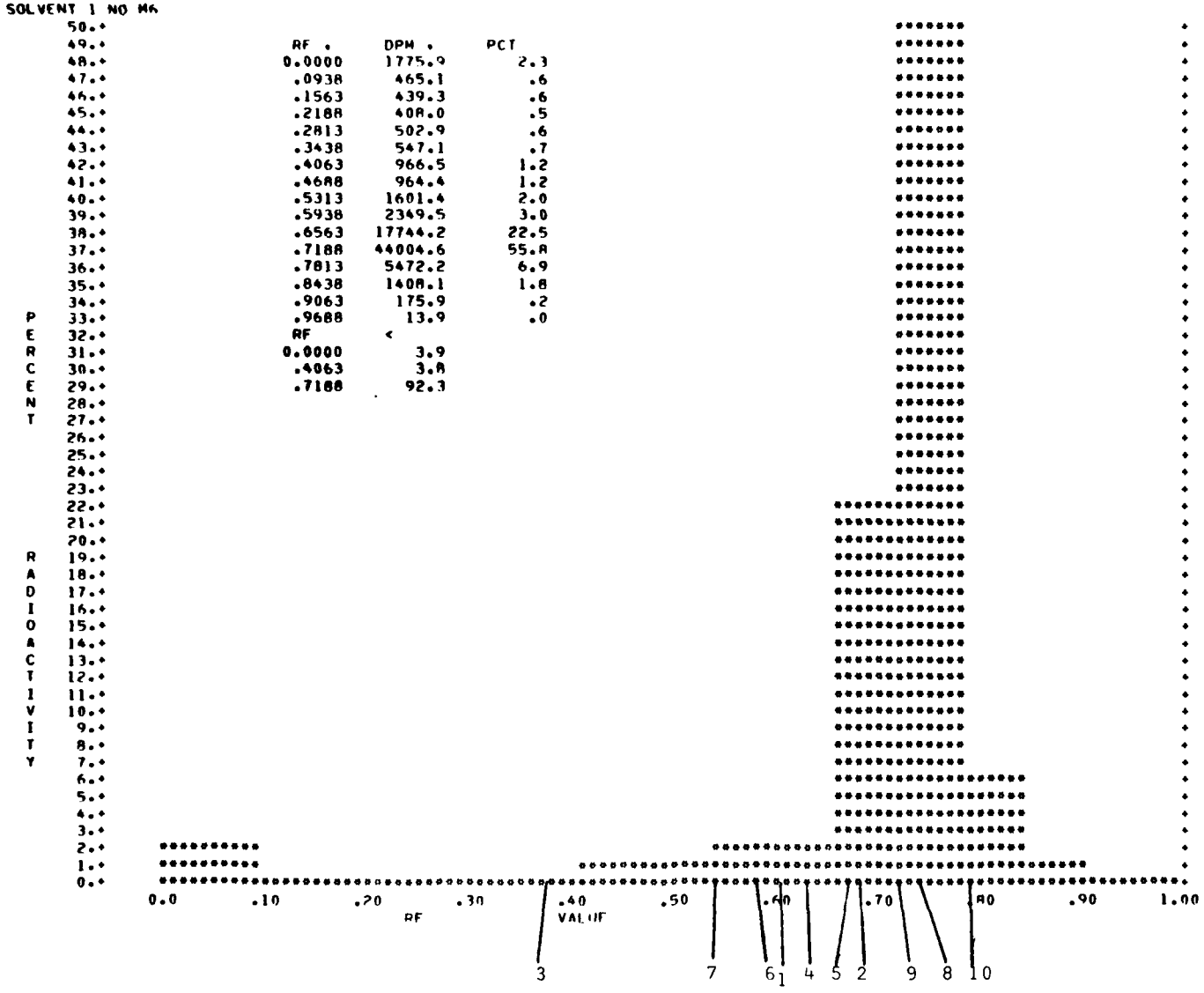


Figure 30-E_6: Solvent I

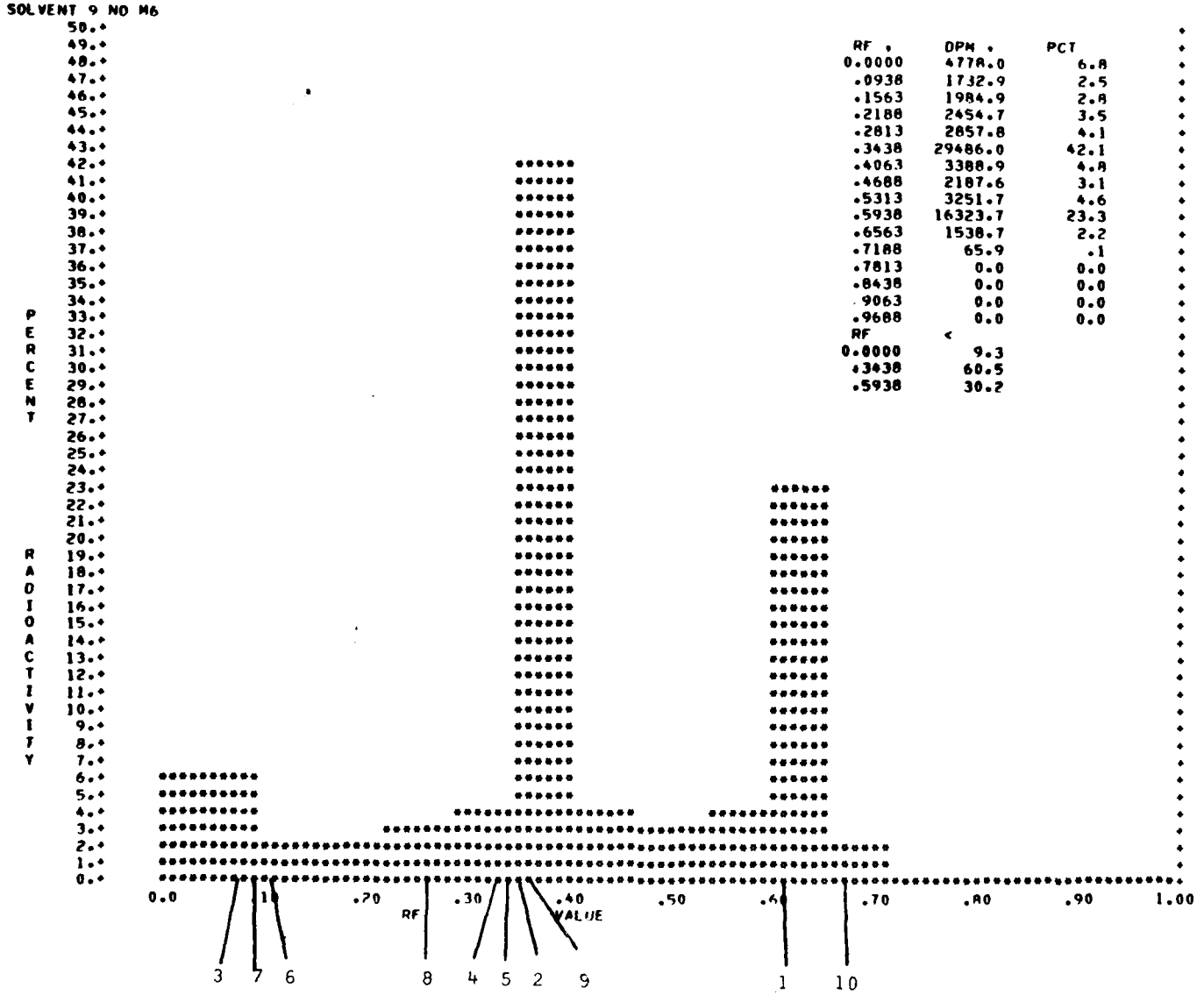


Figure 30-E_6: Solvent IX

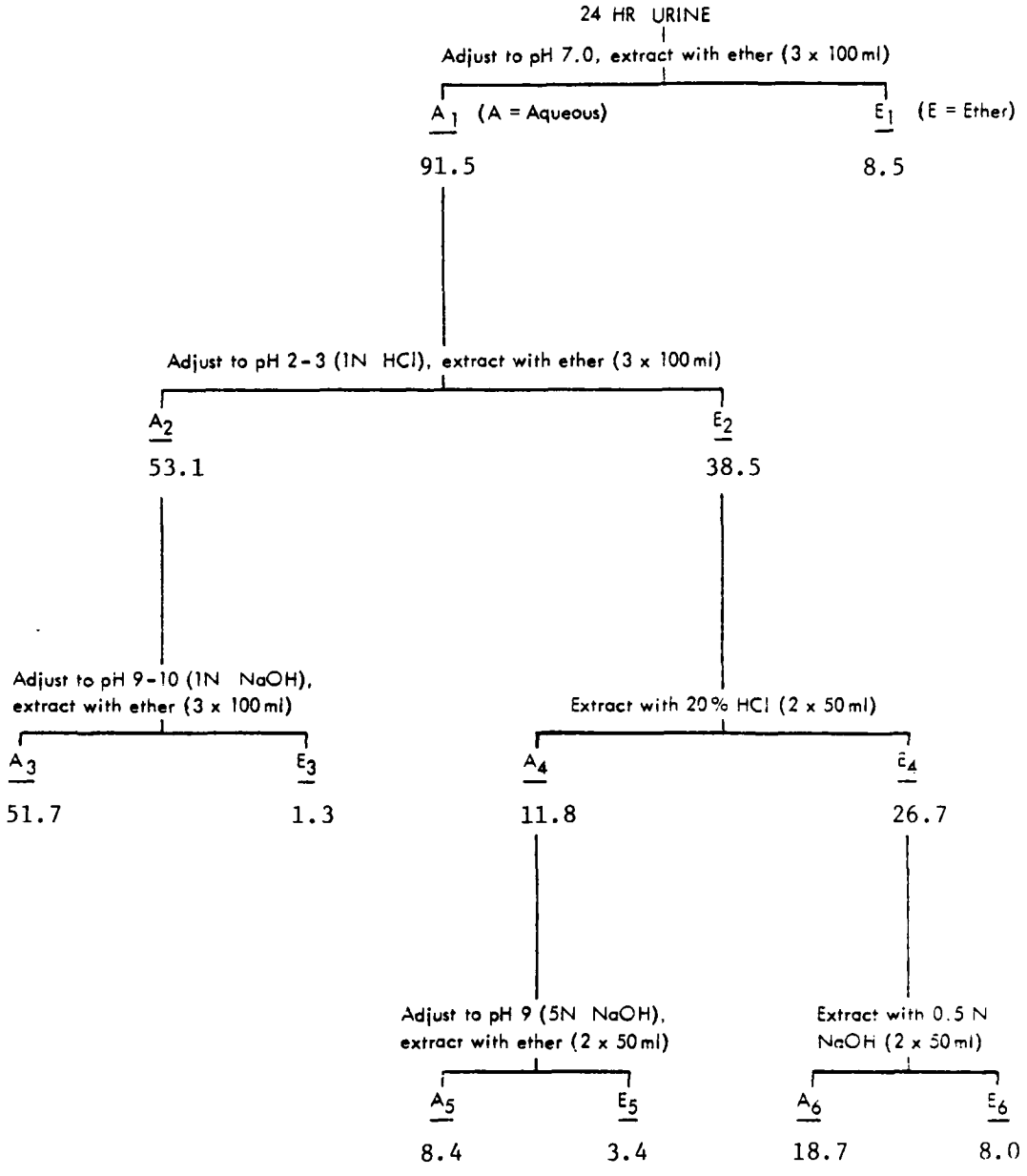


Figure 31: Fractionation of 24-Hr Urine Obtained from Mice Treated Dermally with ^{14}C-TNT. Values indicate the percentage of extractable radioactivity in each fraction.

Figure 32: TLC of Ether-Extractable Products Obtained from 24-Hr Urine of Mice Treated Dermally with ^{14}C-TNT. Extractions were performed at different pH conditions according to the scheme described in the preceeding figure. Solvent I, n-butanol:acetic acid:water, 10L1:1; Solvent IX, toluene:acetic acid, 4:1. For reference metabolites (1-10) see Figure 26 or Table 19. Reference metabolites are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzyl alcohol
3. Trinitrobenzoic Acid
4. 4-Amino-2,6-dinitrotoluene
5. 2-Amino-4,6-dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2',6'-Tetranitro-4,4'-azoxytoluene

Figure 32 follows

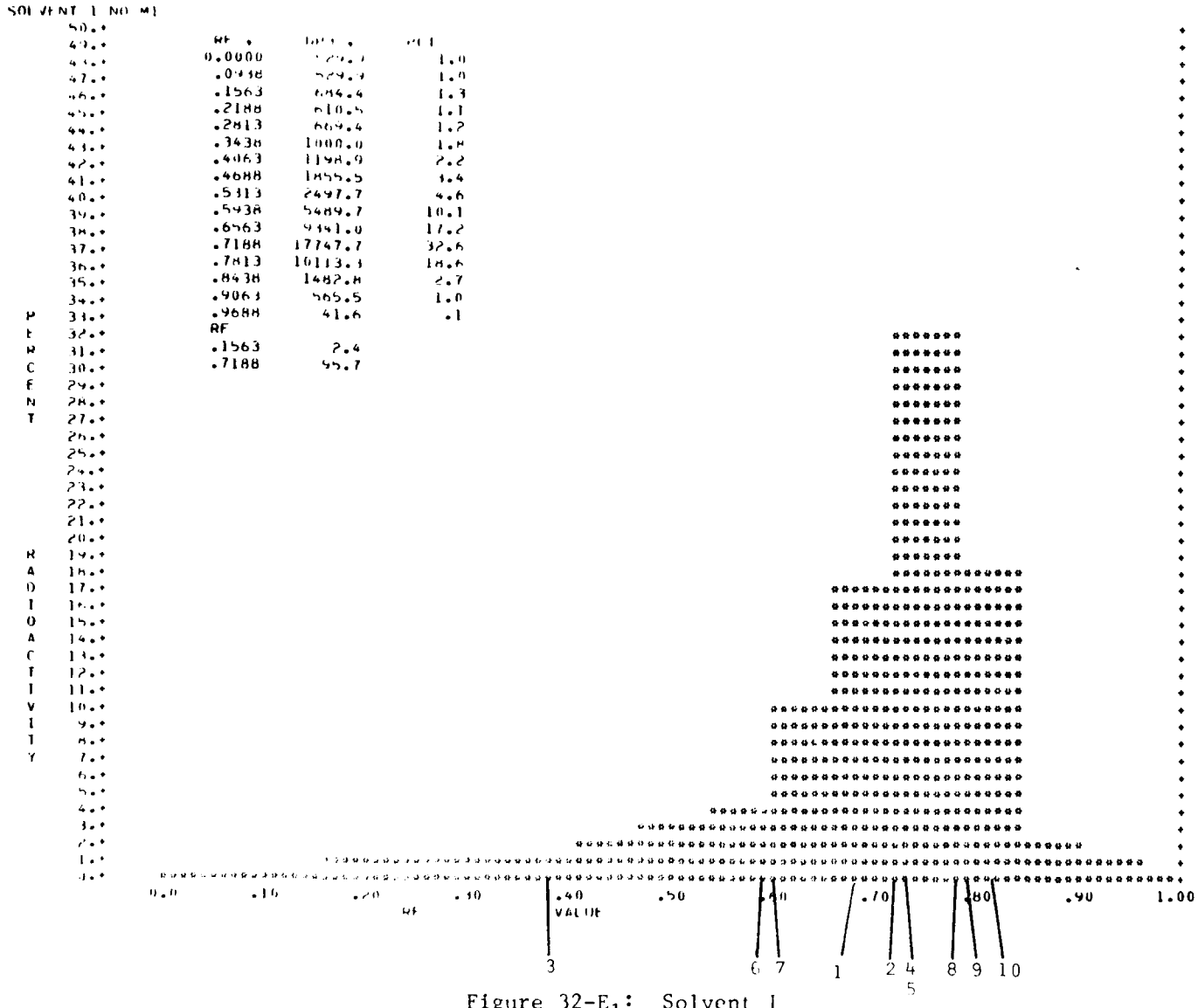


Figure 32-E_1: Solvent 1

AD-A114 025 MIDWEST RESEARCH INST KANSAS CITY MO F/G 6/20
SPECIES DIFFERENCES IN THE DISPOSITION AND METABOLISM OF 2,4,6---ETC(U)
JUN 81 A M EL-HAWARI, J R HODGSON DAMD17-76-C-6066
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5 OF 5
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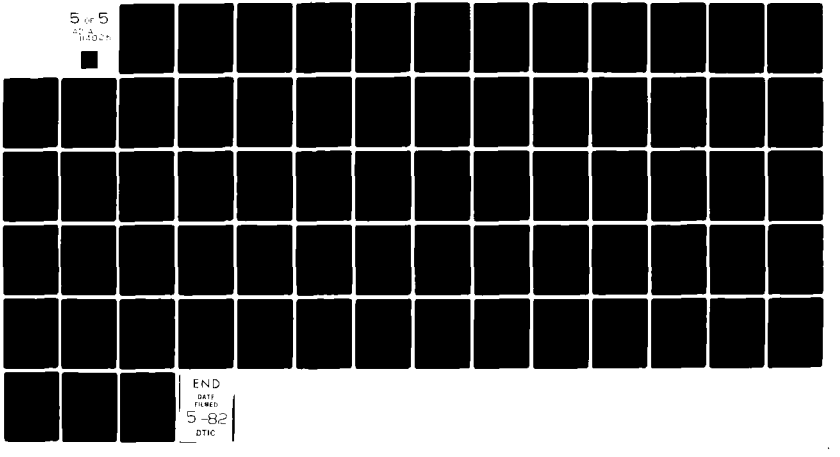

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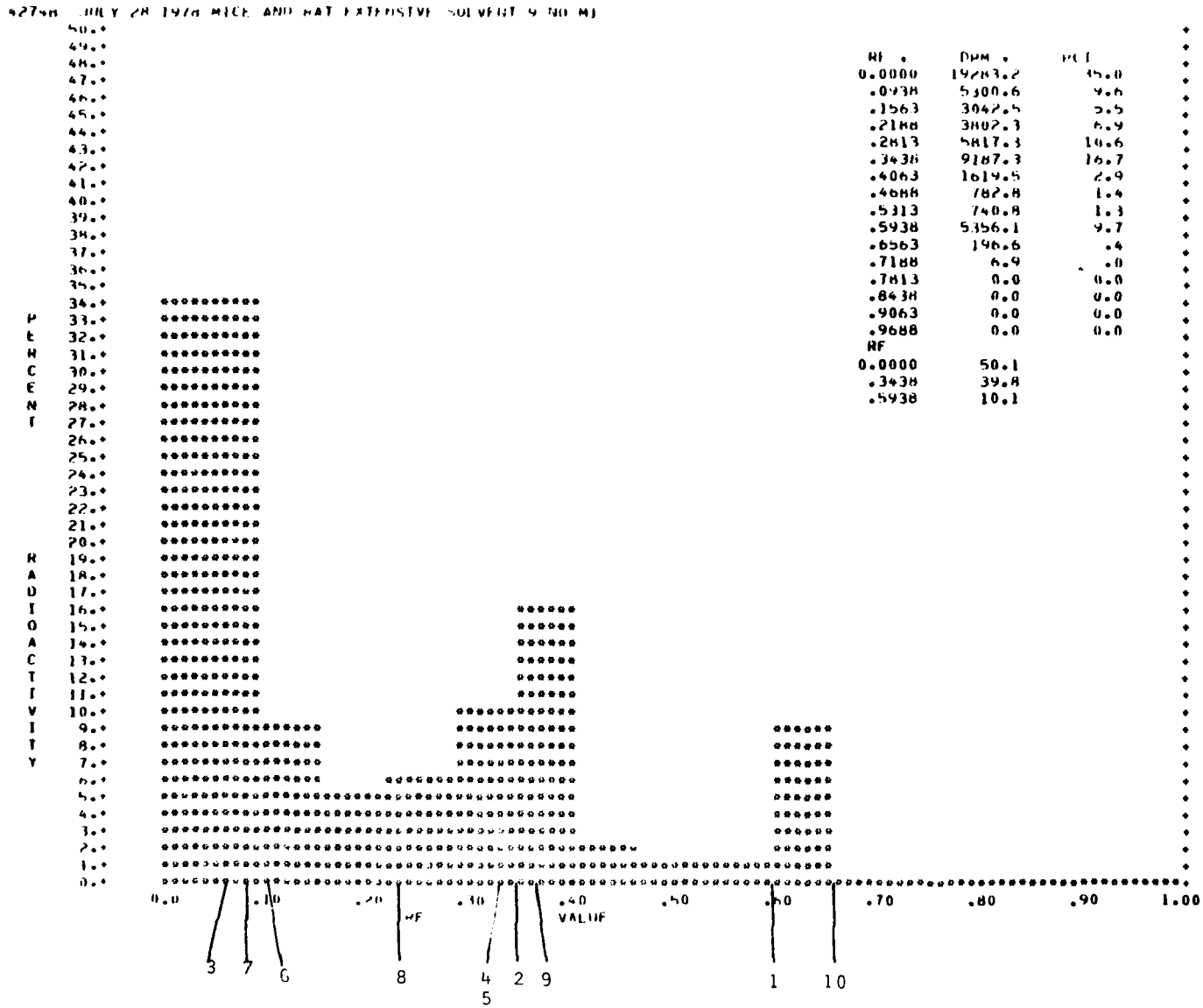

Figure 32-E_1: Solvent IX

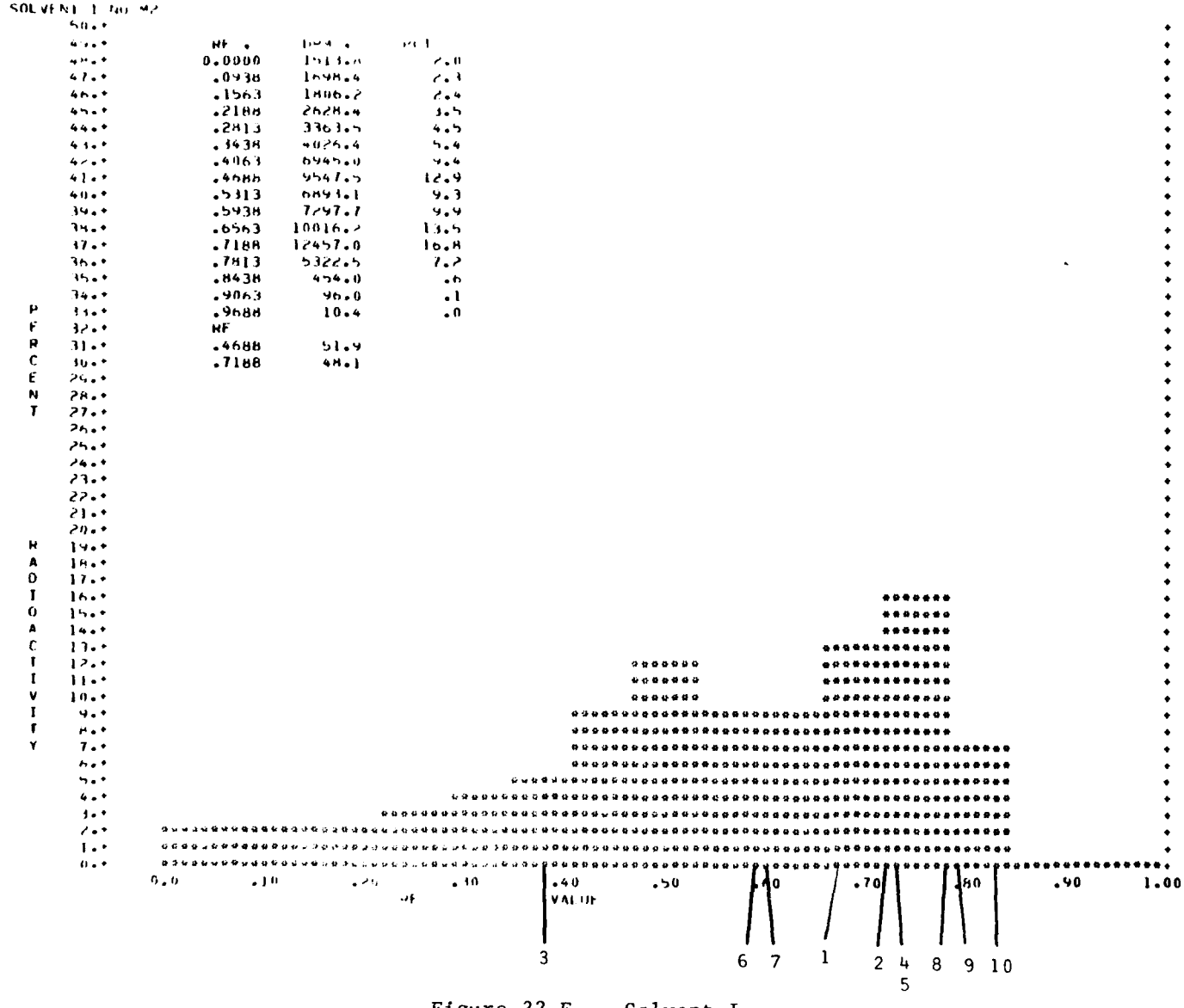

Figure 32-E_2: Solvent I

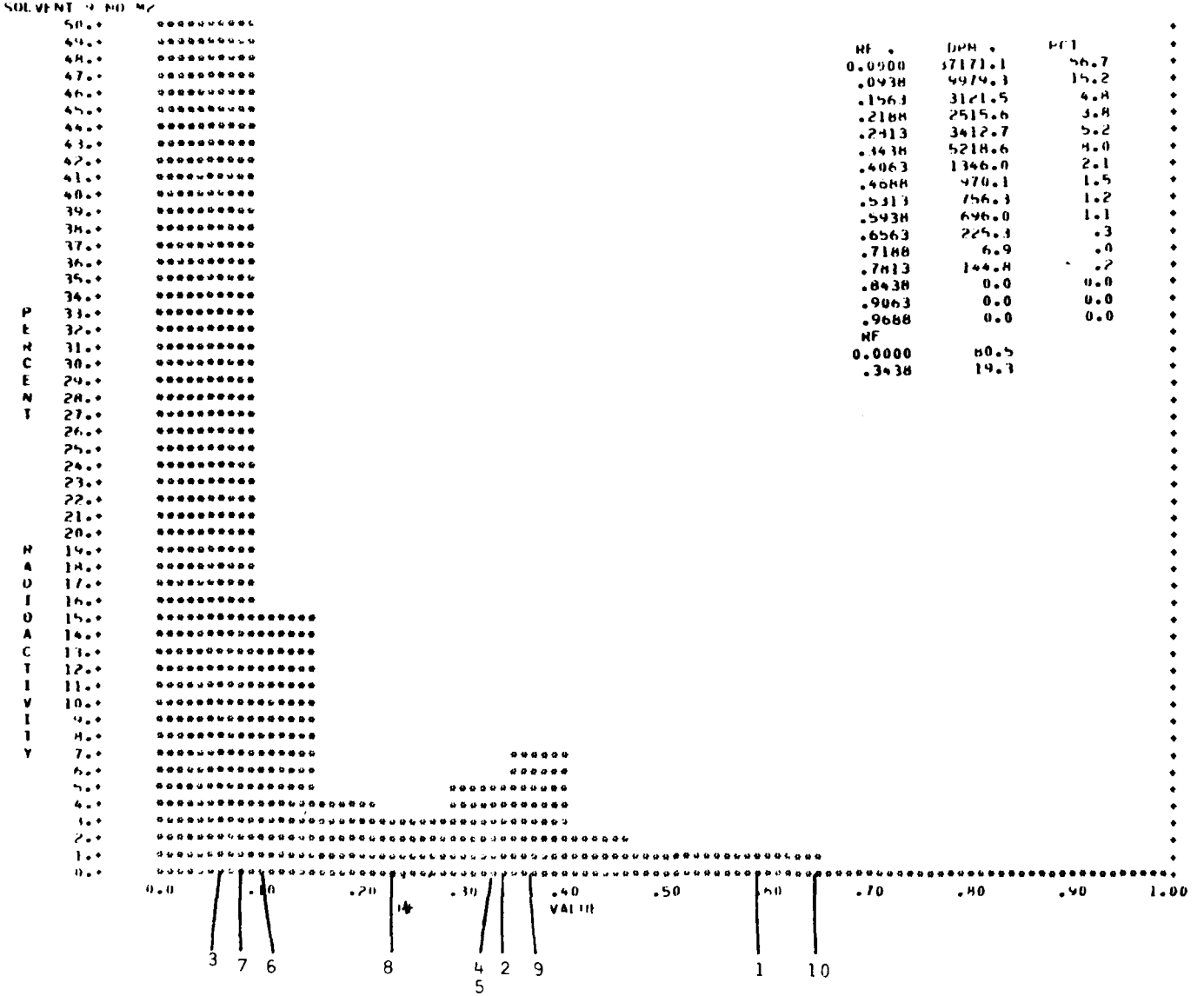

Figure 32-E_2: Solvent IX

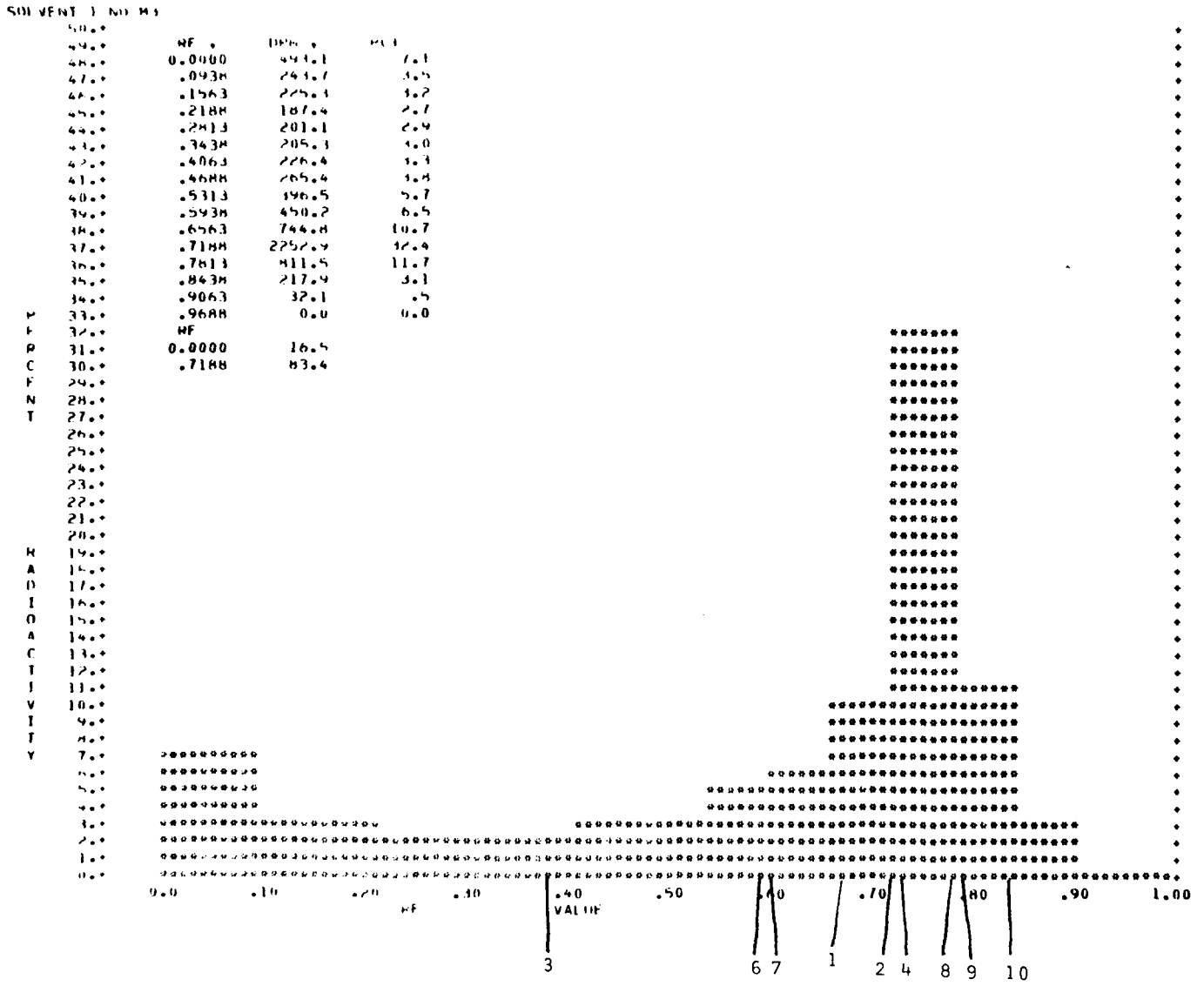

Figure 32-E_3: Solvent I

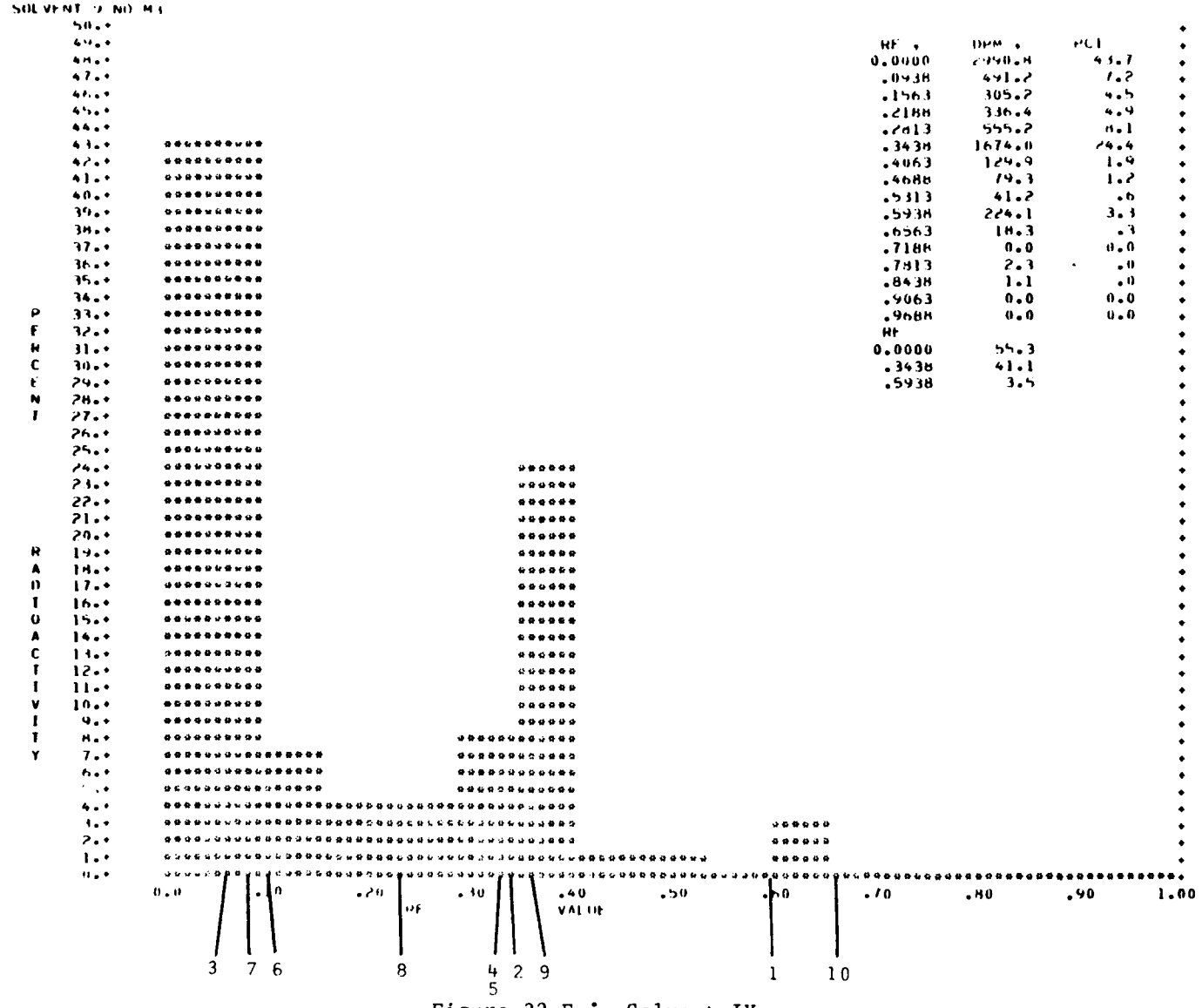

Figure 32-E_3: Solvent IX

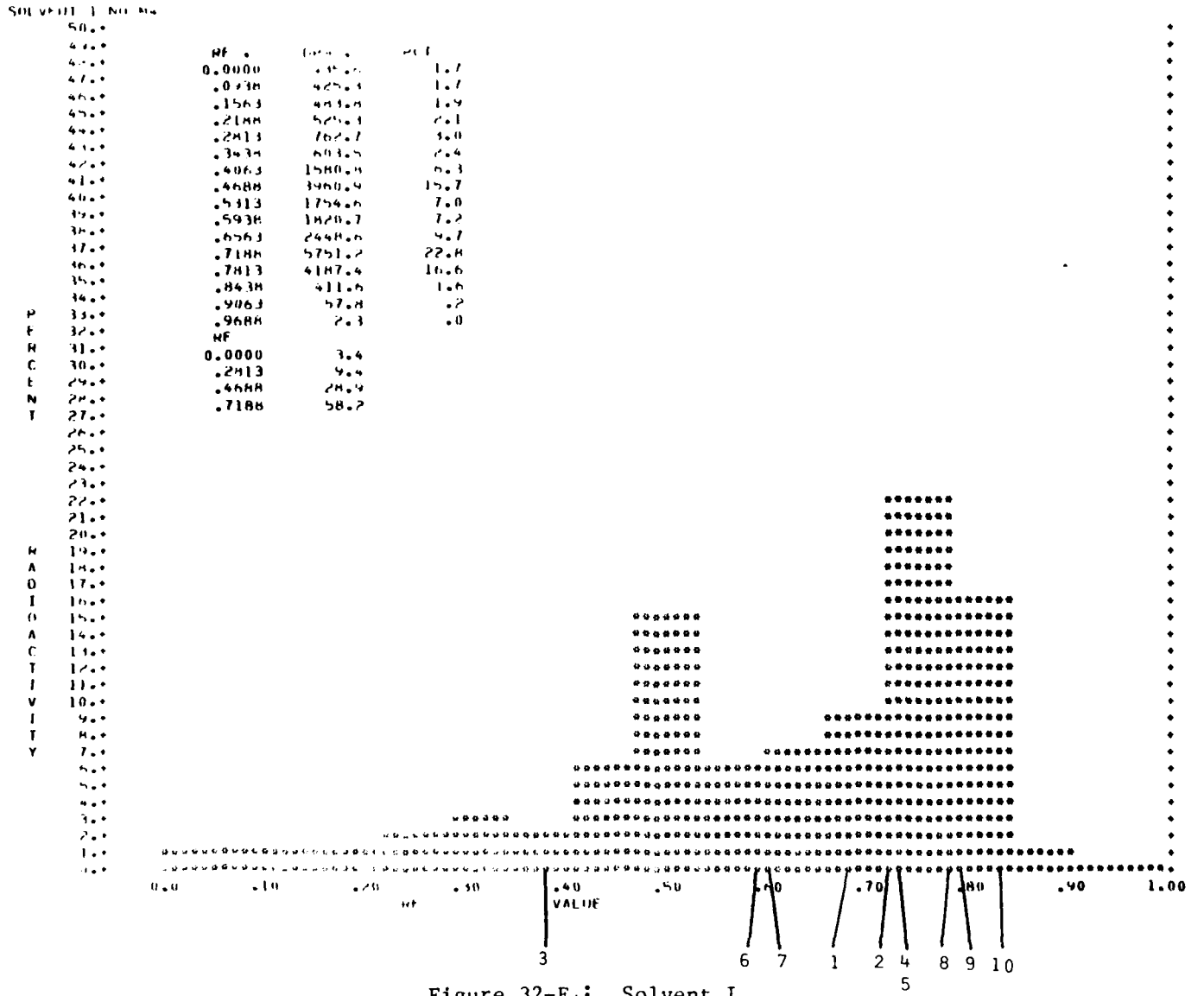

Figure 32-E_4: Solvent I

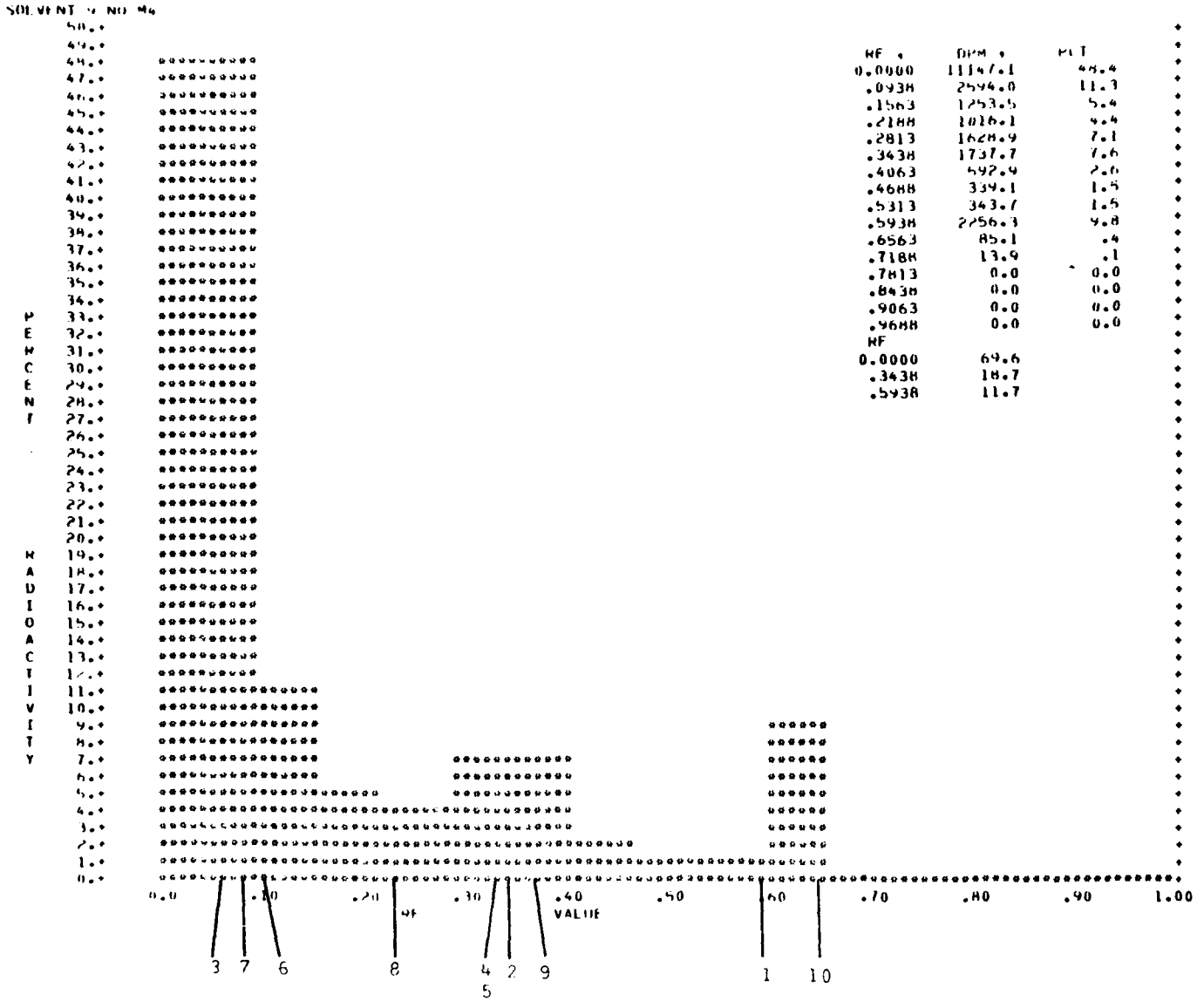

Figure 32-E_4: Solvent IX

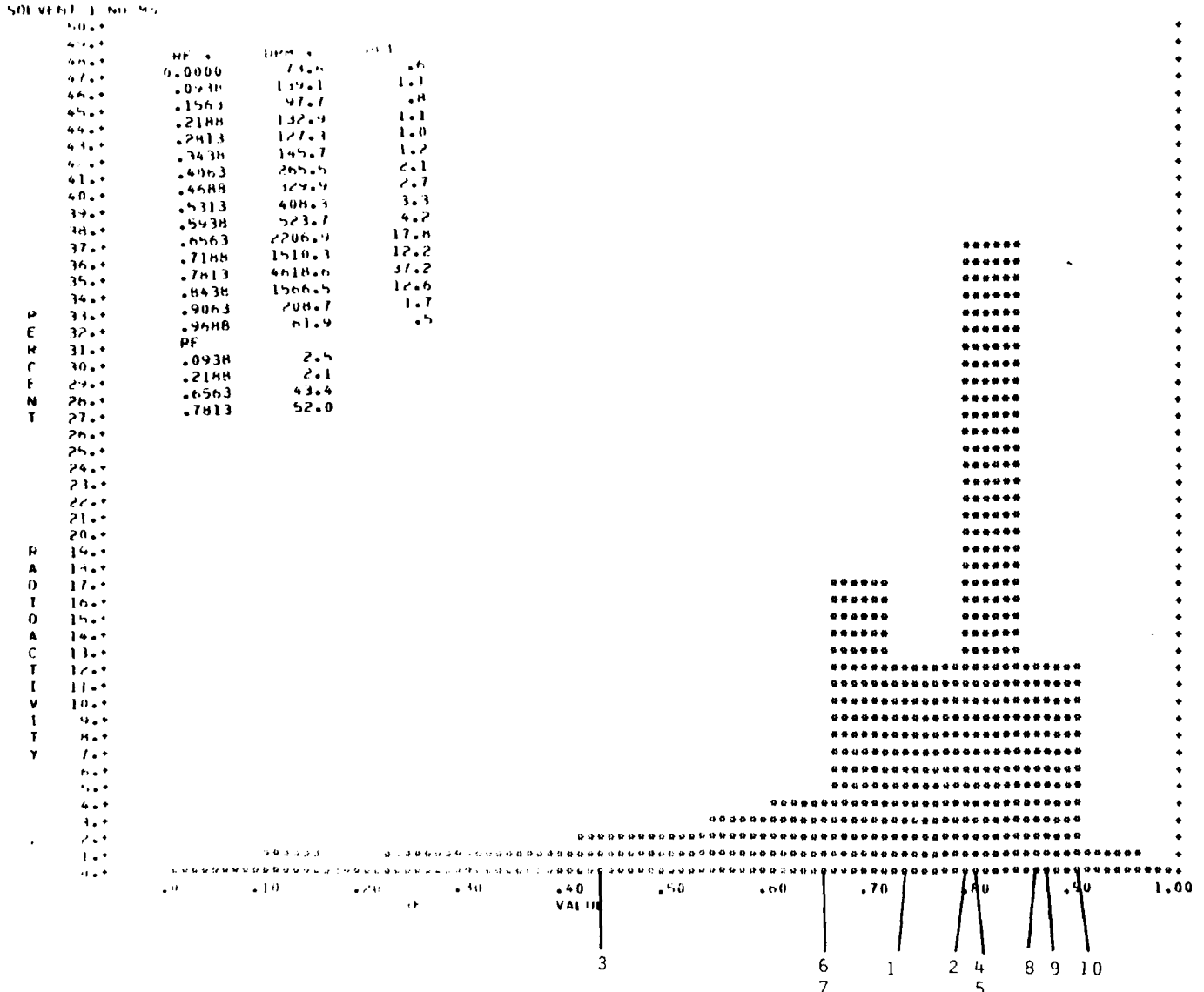

Figure 32-E_5: Solvent I

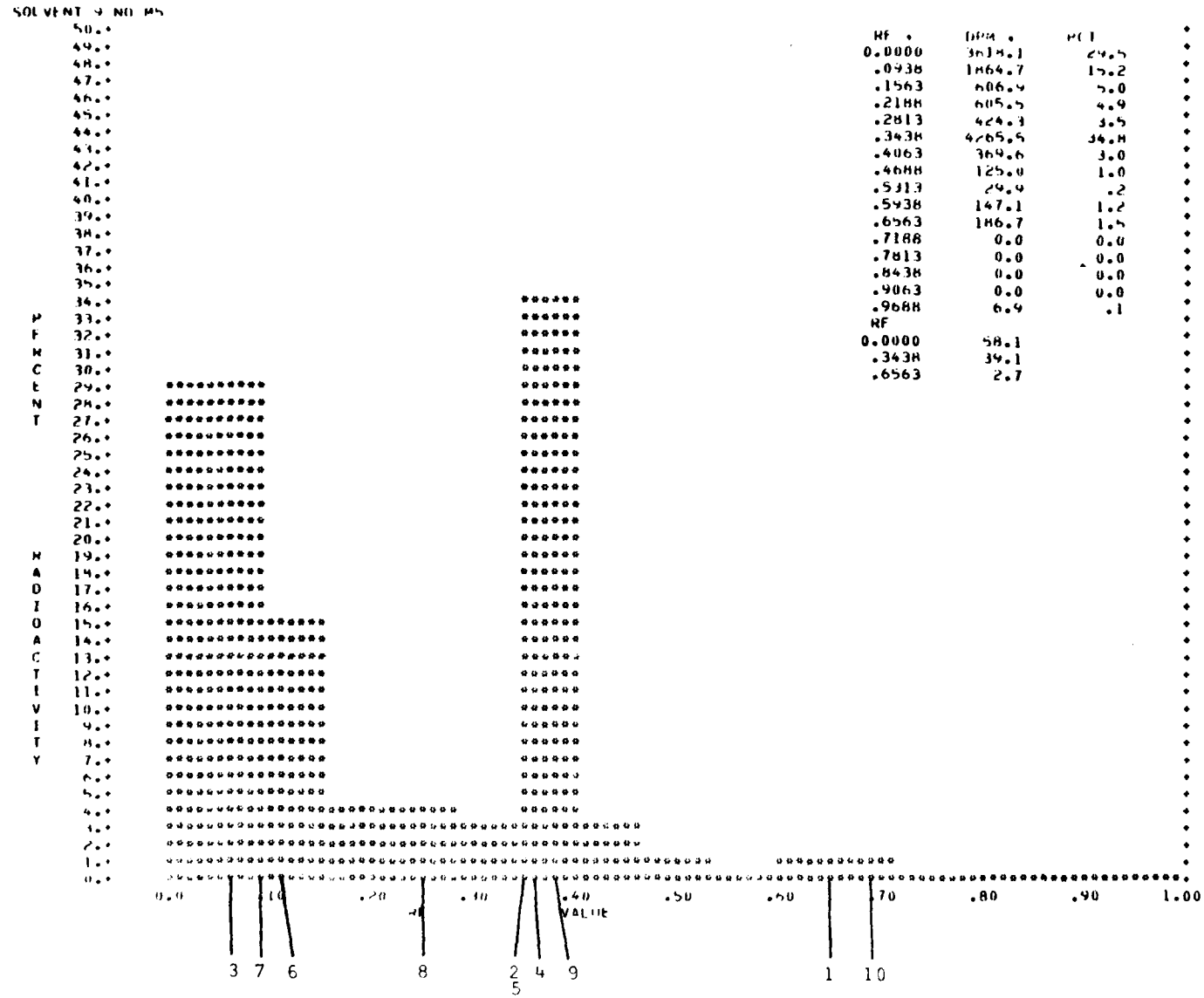

Figure 32-E_5: Solvent IX

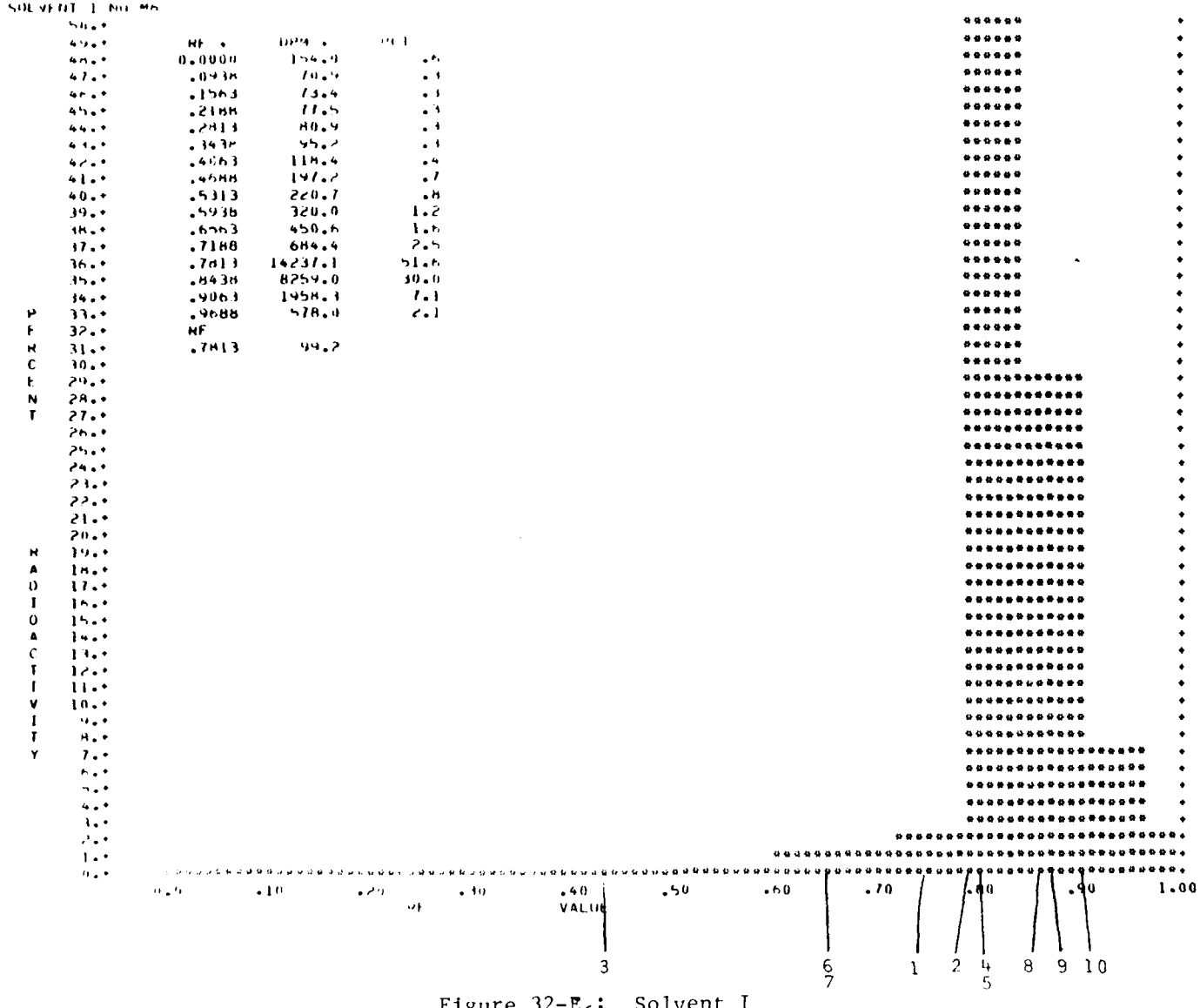

Figure 32-E_6: Solvent I

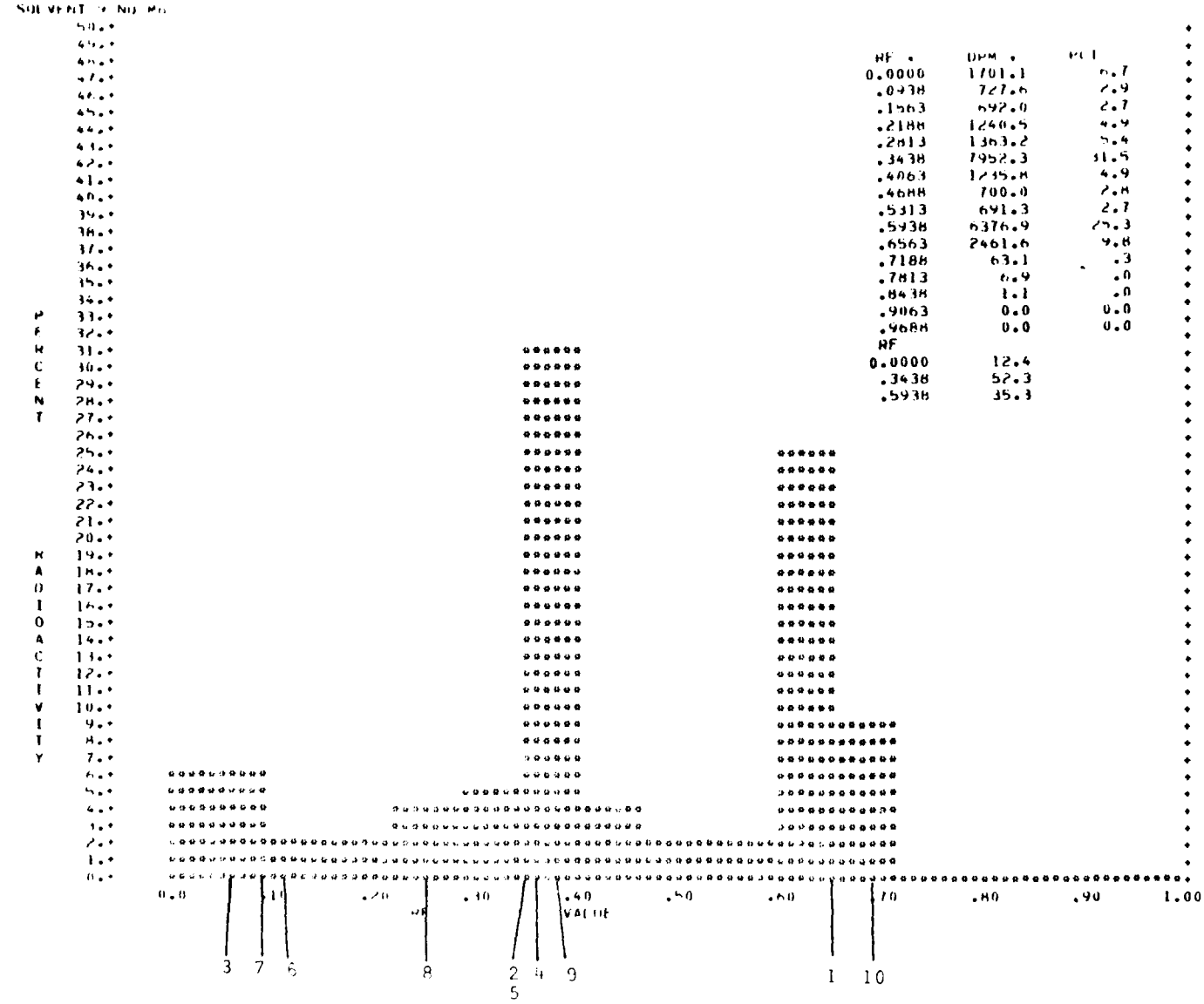

Figure 32-E_6: Solvent IX

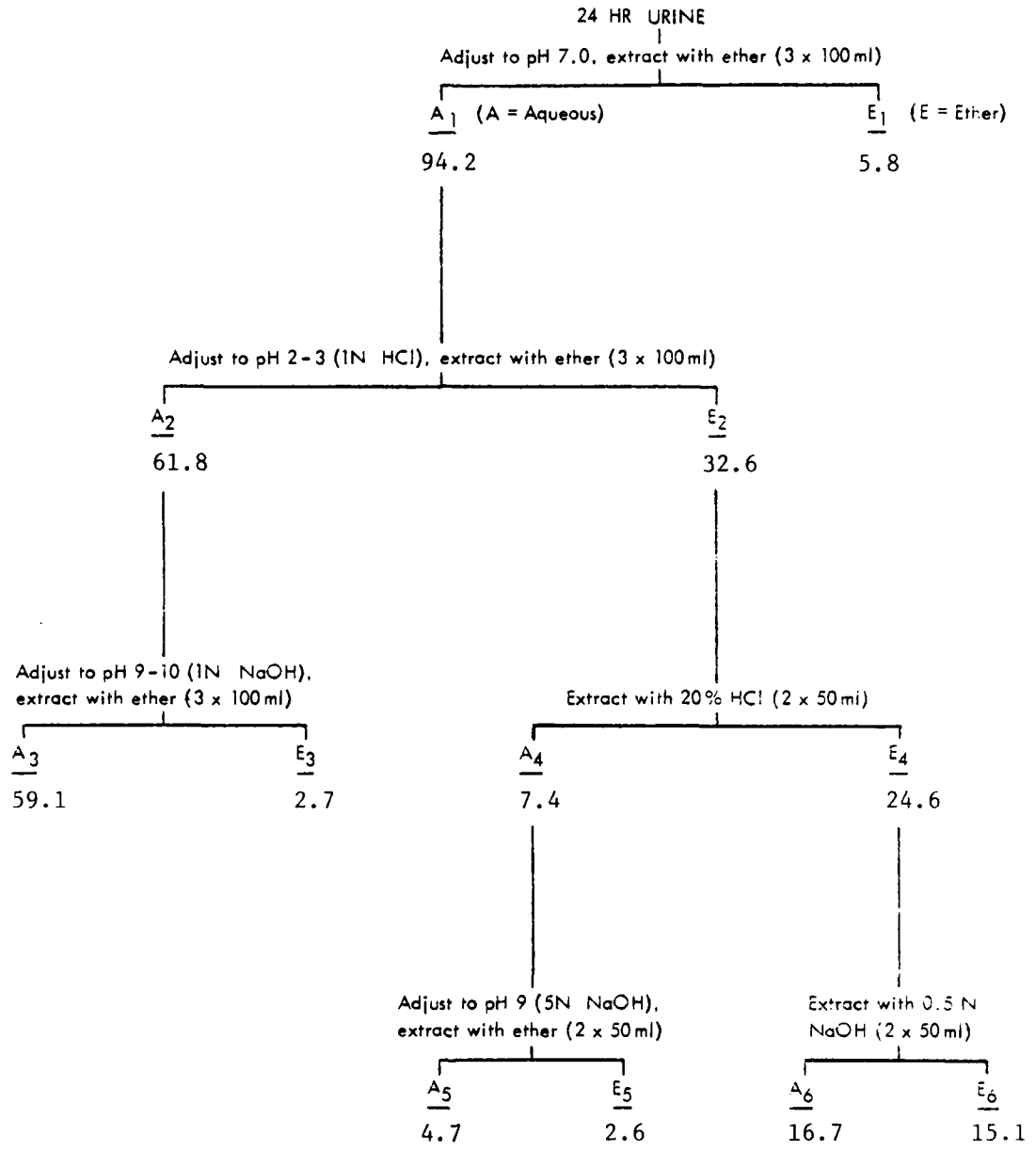

Figure 33: Fractionation of 24-Hr Urine Obtained from Rabbits Treated Orally with ^{14}C-TNT. Values indicate the percentage of extractable radioactivity in each fraction.

Figure 34, E_1-6_6: TLC of Ether-Extractable Products Obtained from 24-Hr Urine of Rabbits Treated Orally with ^{14}C-TNT. Extractions were performed at different pH conditions according to the scheme described in the preceding figure. Solvent I, n-butanol:acetic acid: water, 10:1:1; Solvent IX, toluene:acetic acid, 4:1. (For reference metabolites (1-10) see Figure 26 or Table 19.) Reference metabolites are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzyl alcohol
3. Trinitrobenzoic Acid
4. 4-Amino-2,6-dinitrotoluene
5. 2-Amino-4,6-dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2',6'-Tetranitro-4,4'-azoxytoluene

Figure 34 follows

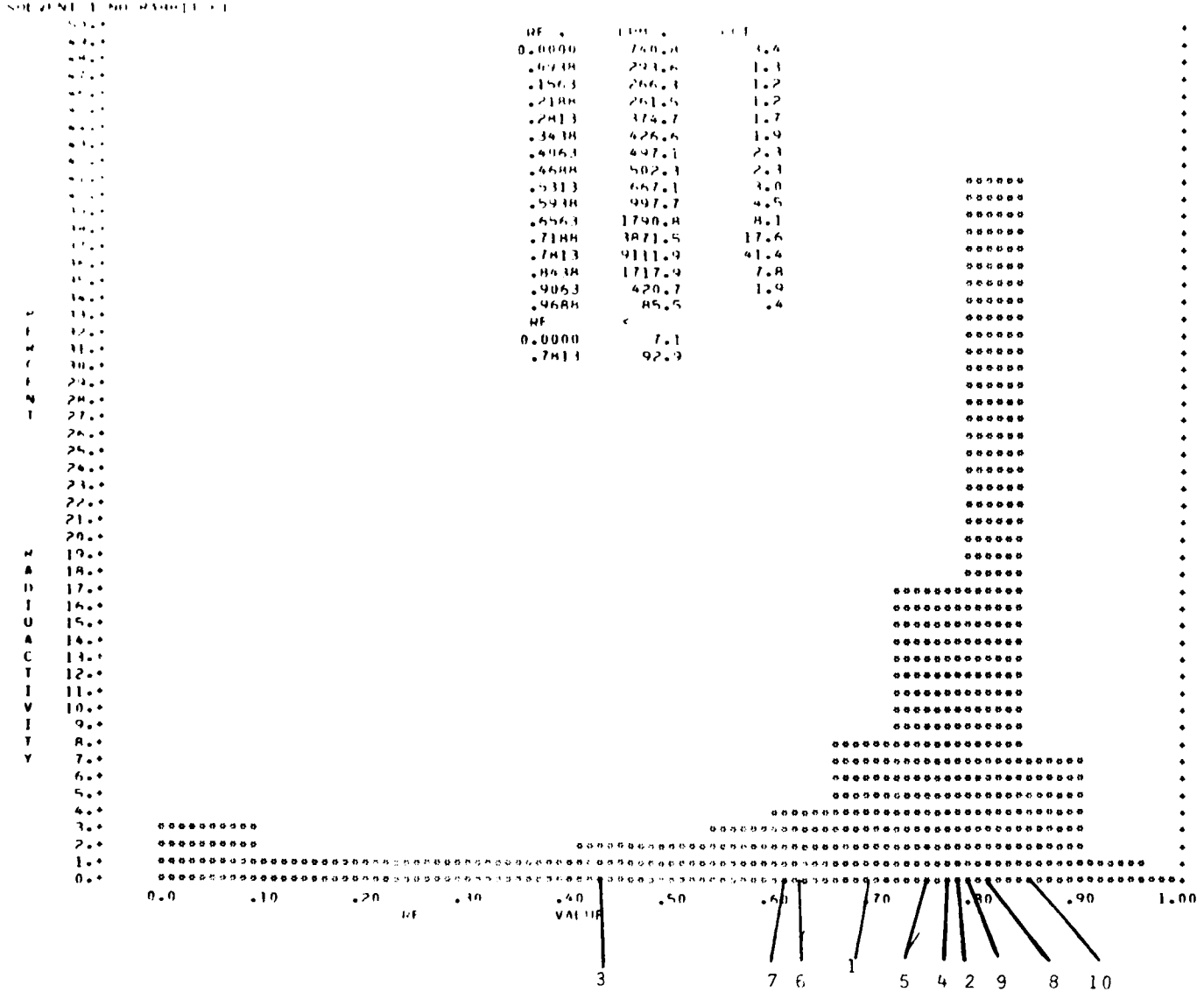

Figure 34-E_1: Solvent I

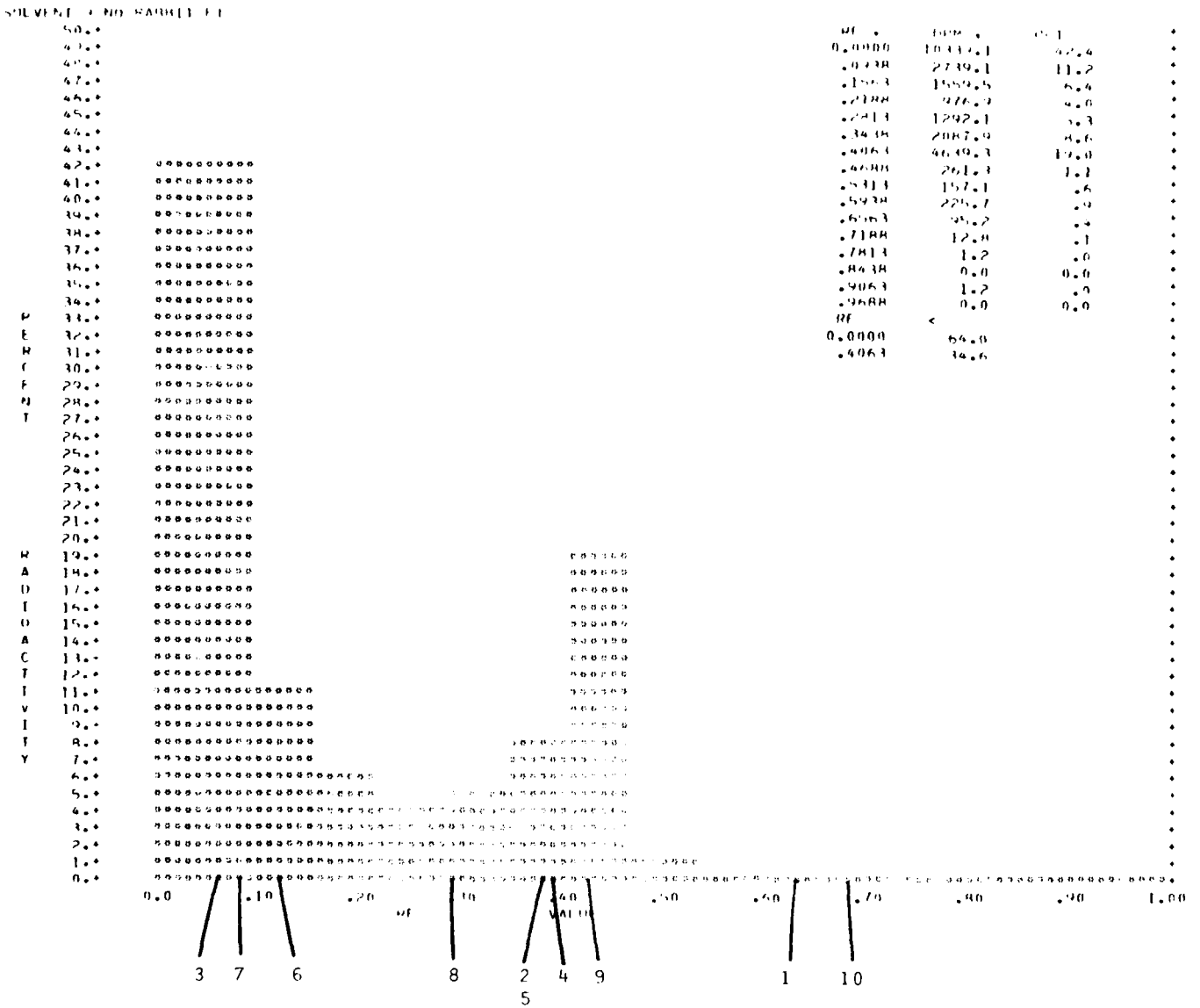

Figure 34-E_1: Solvent IX

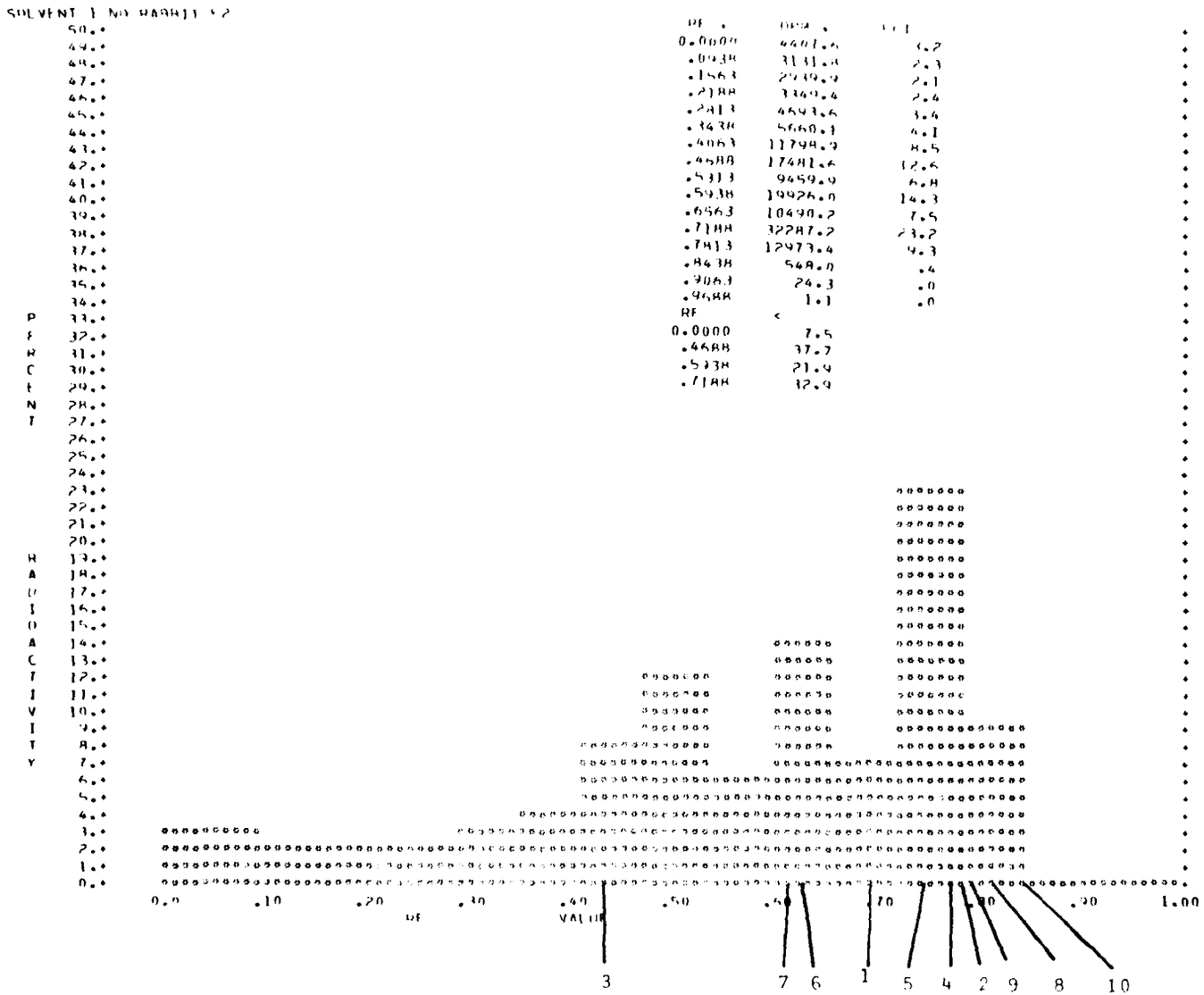

Figure 34-E_2: Solvent I

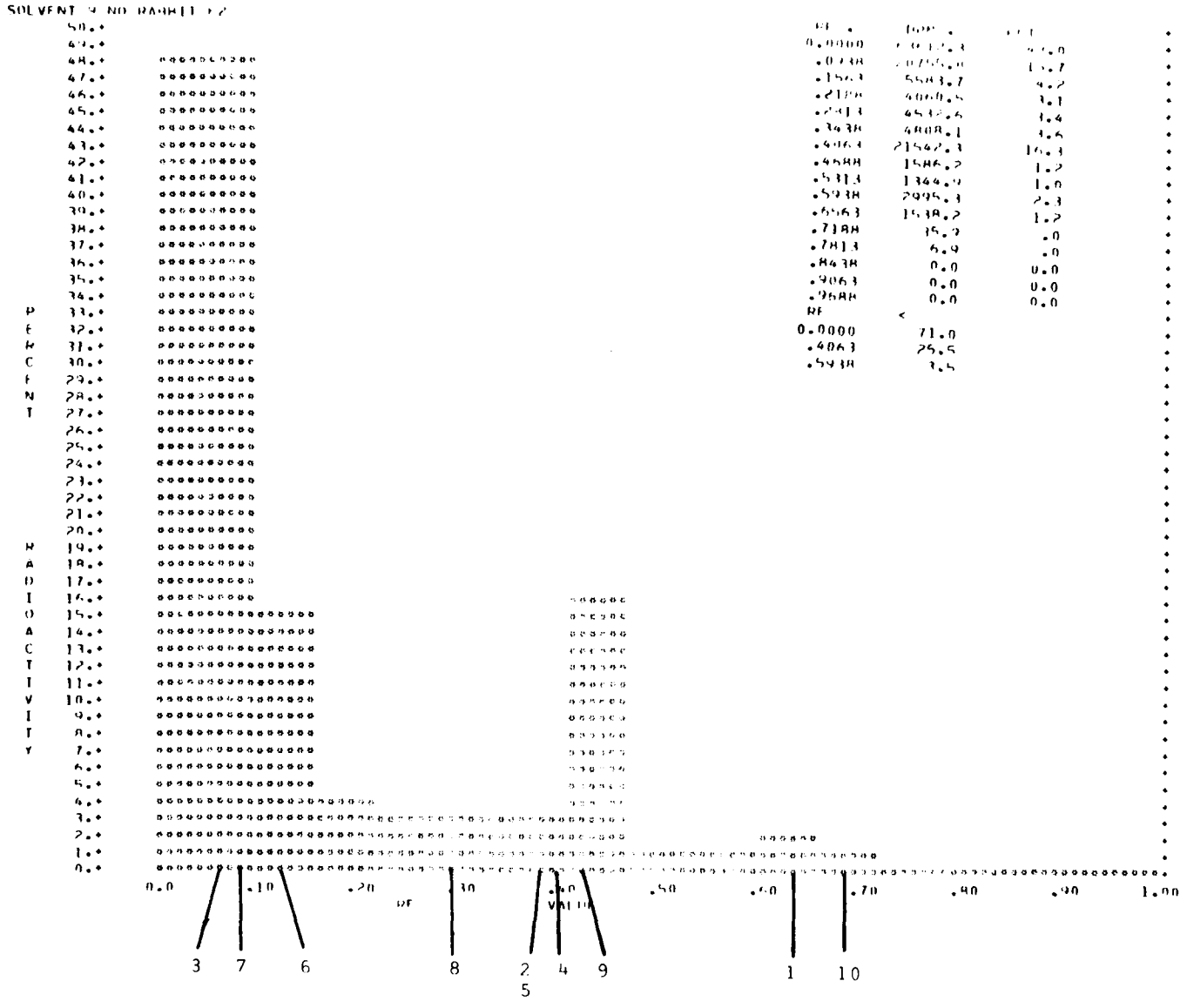

Figure 34-E_2: Solvent IX

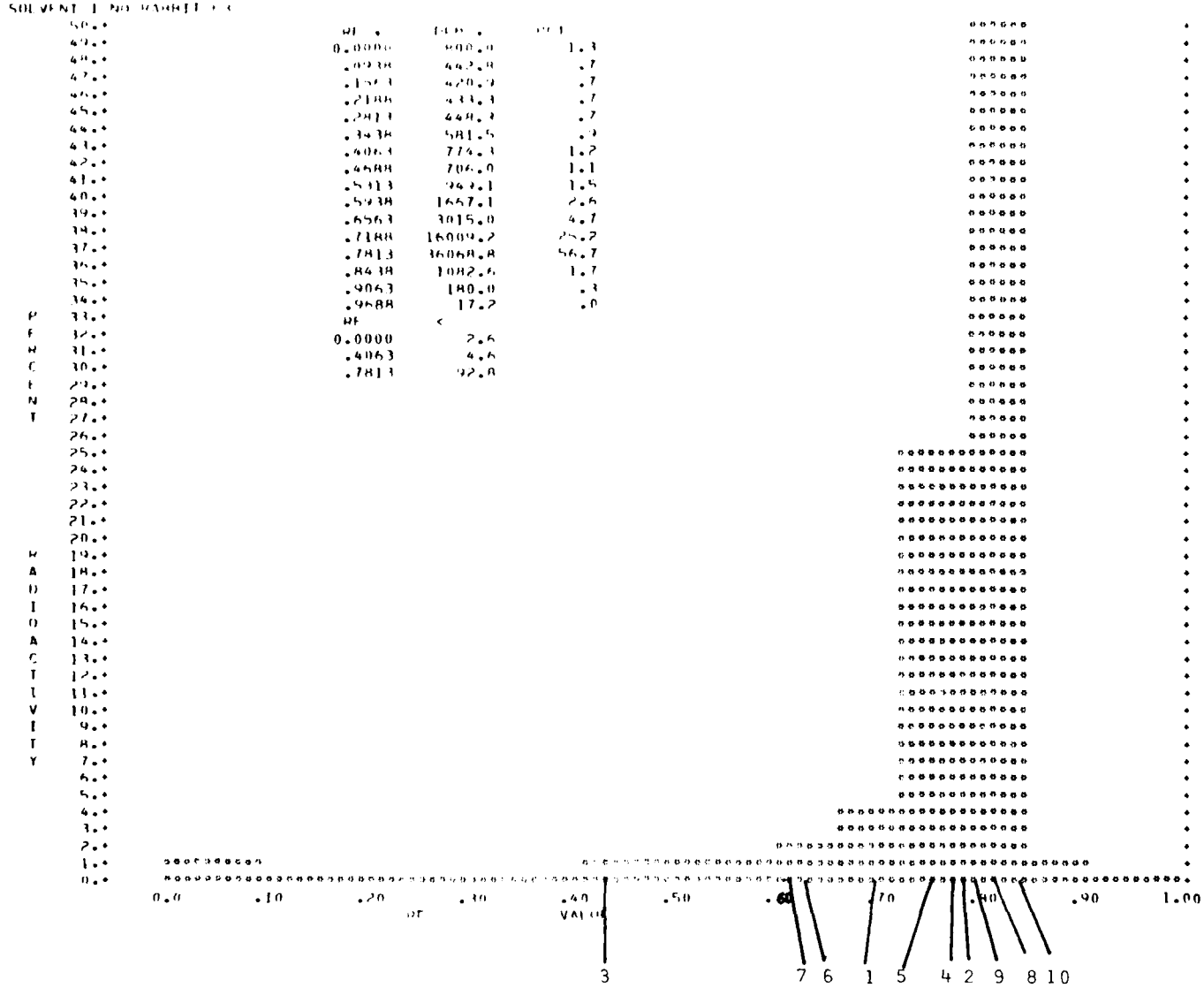

Figure 34-E3: Solvent I

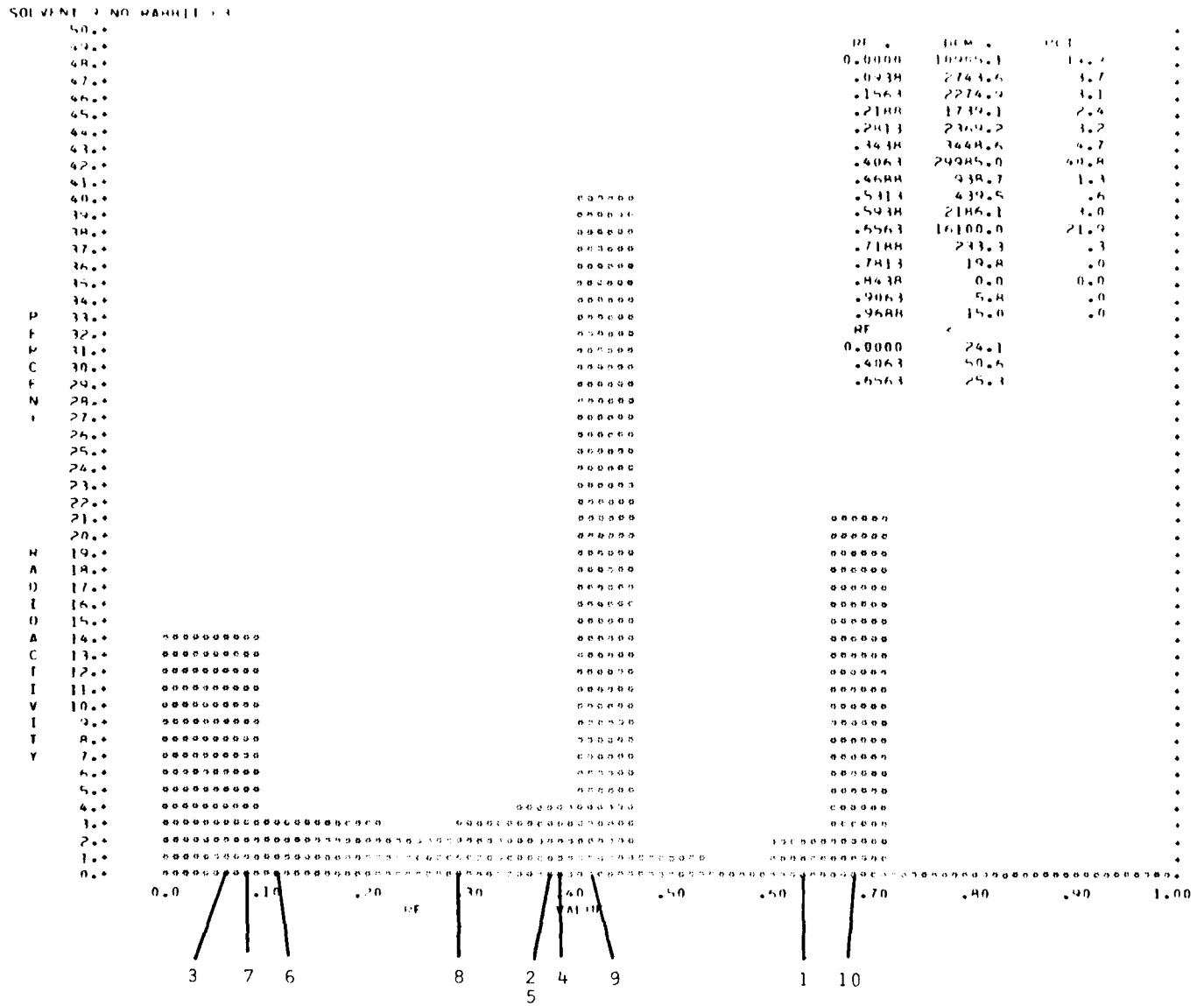

Figure 34-E3: Solvent IX

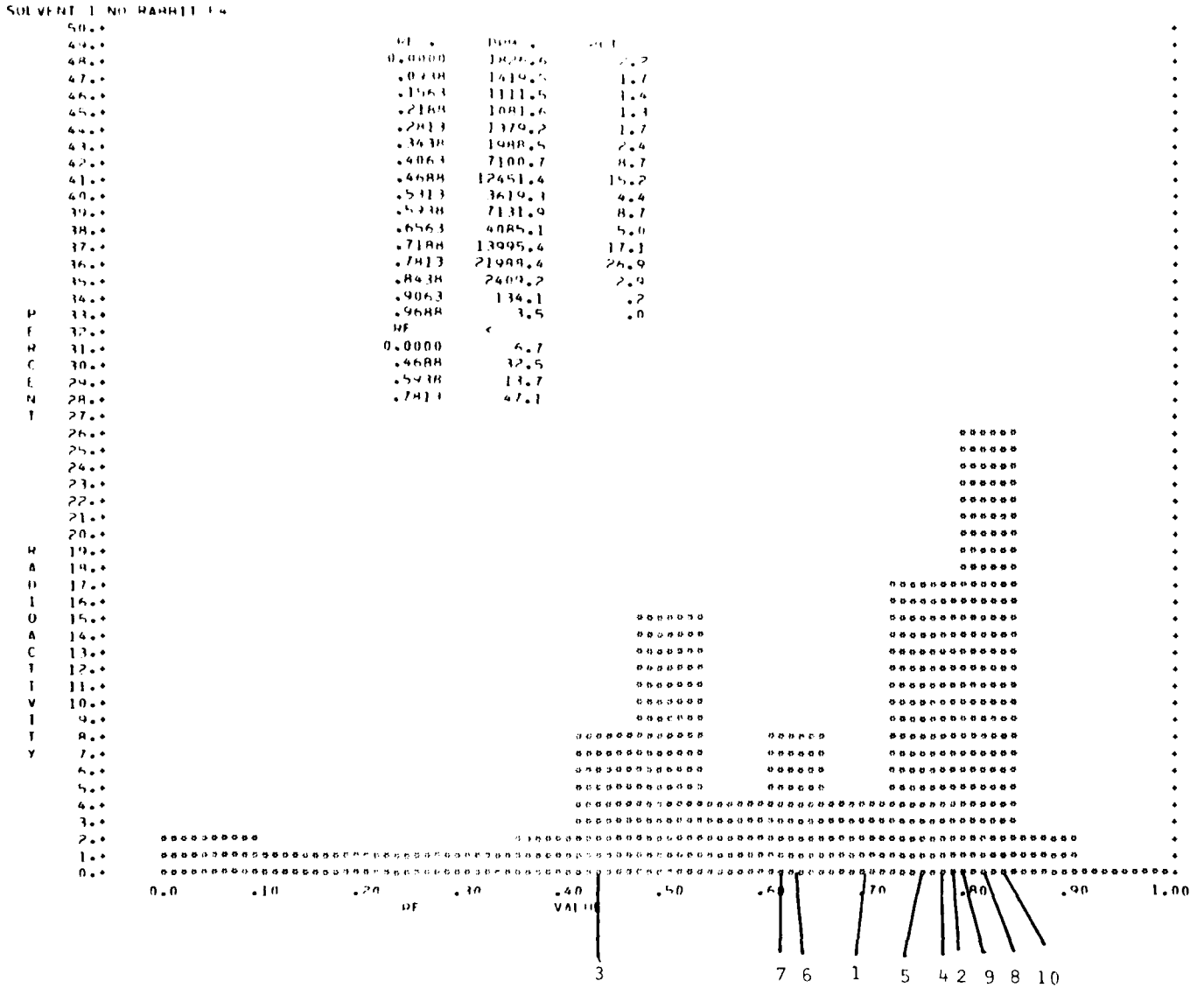

Figure 34-E_4: Solvent I

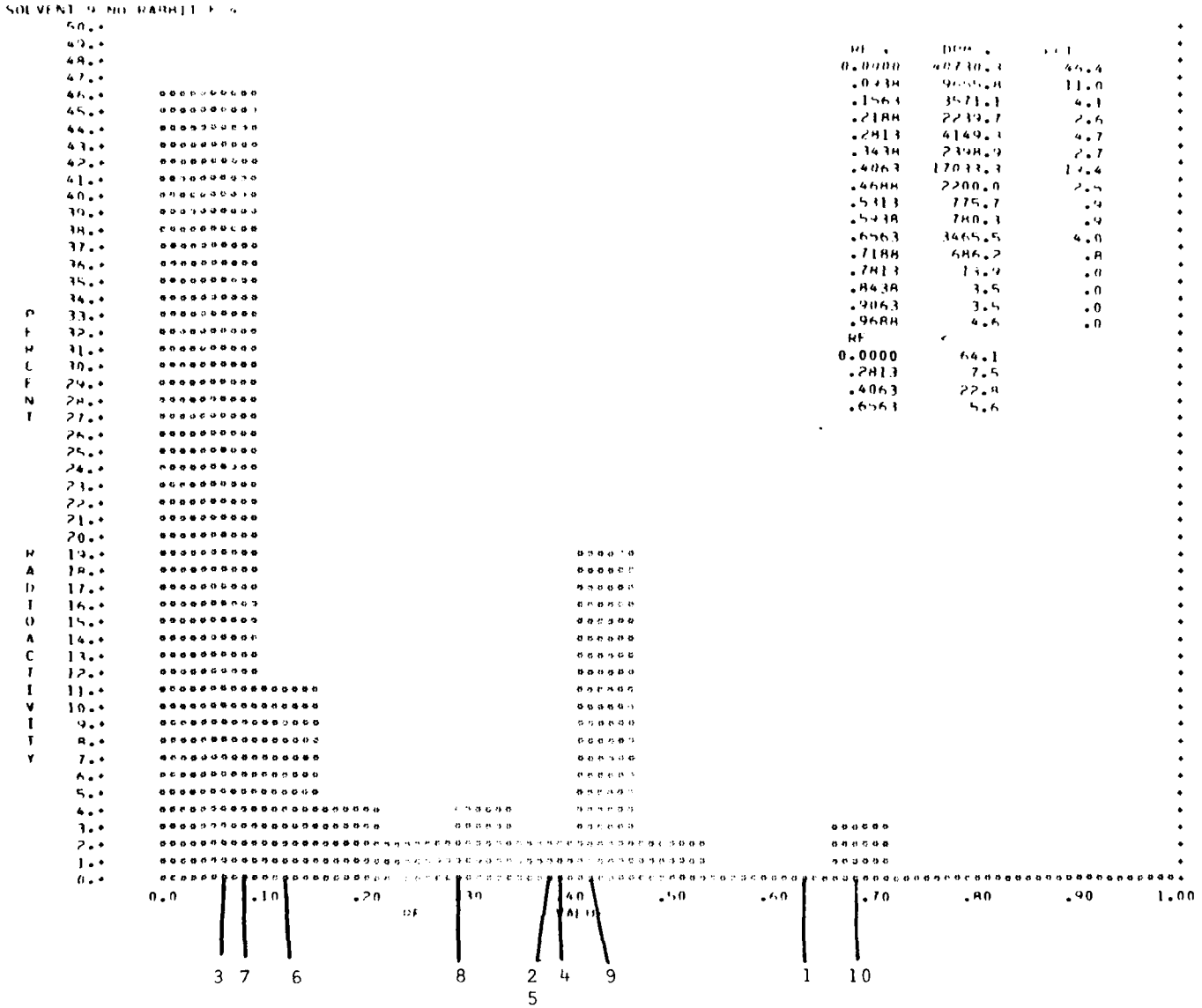

Figure 34-E_4: Solvent IX

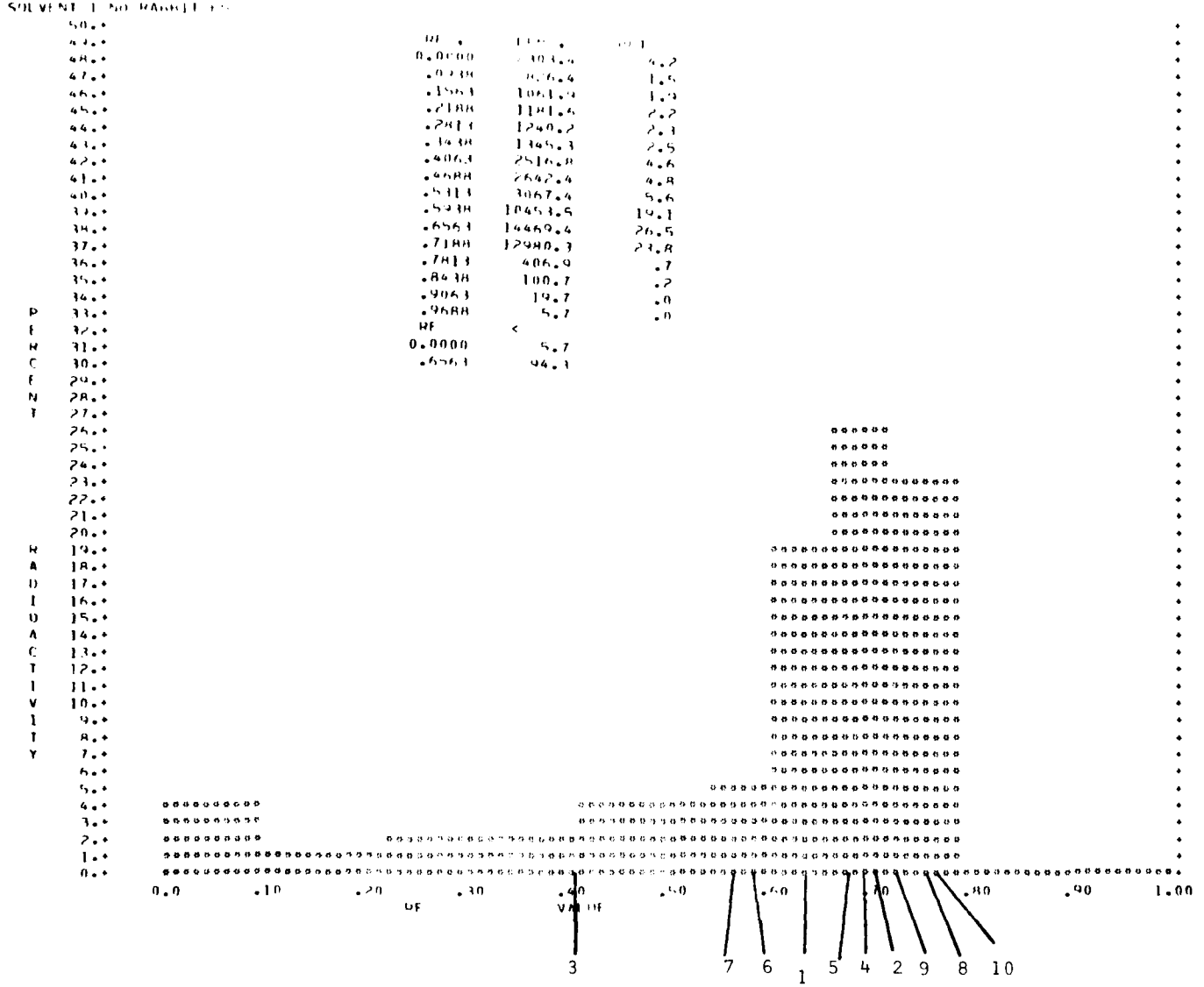

Figure 34-E_5: Solvent I

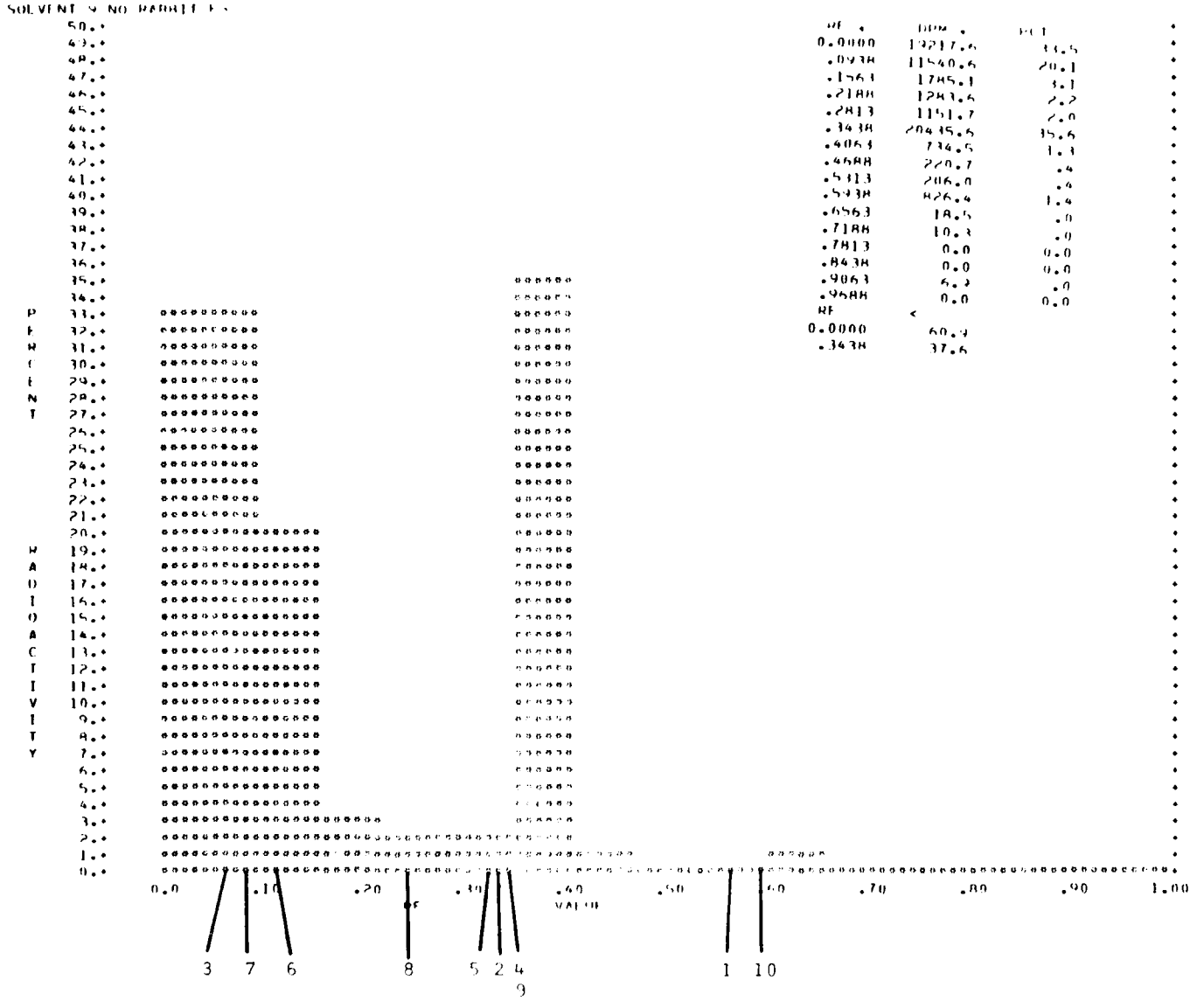

Figure 34-E_5: Solvent IX

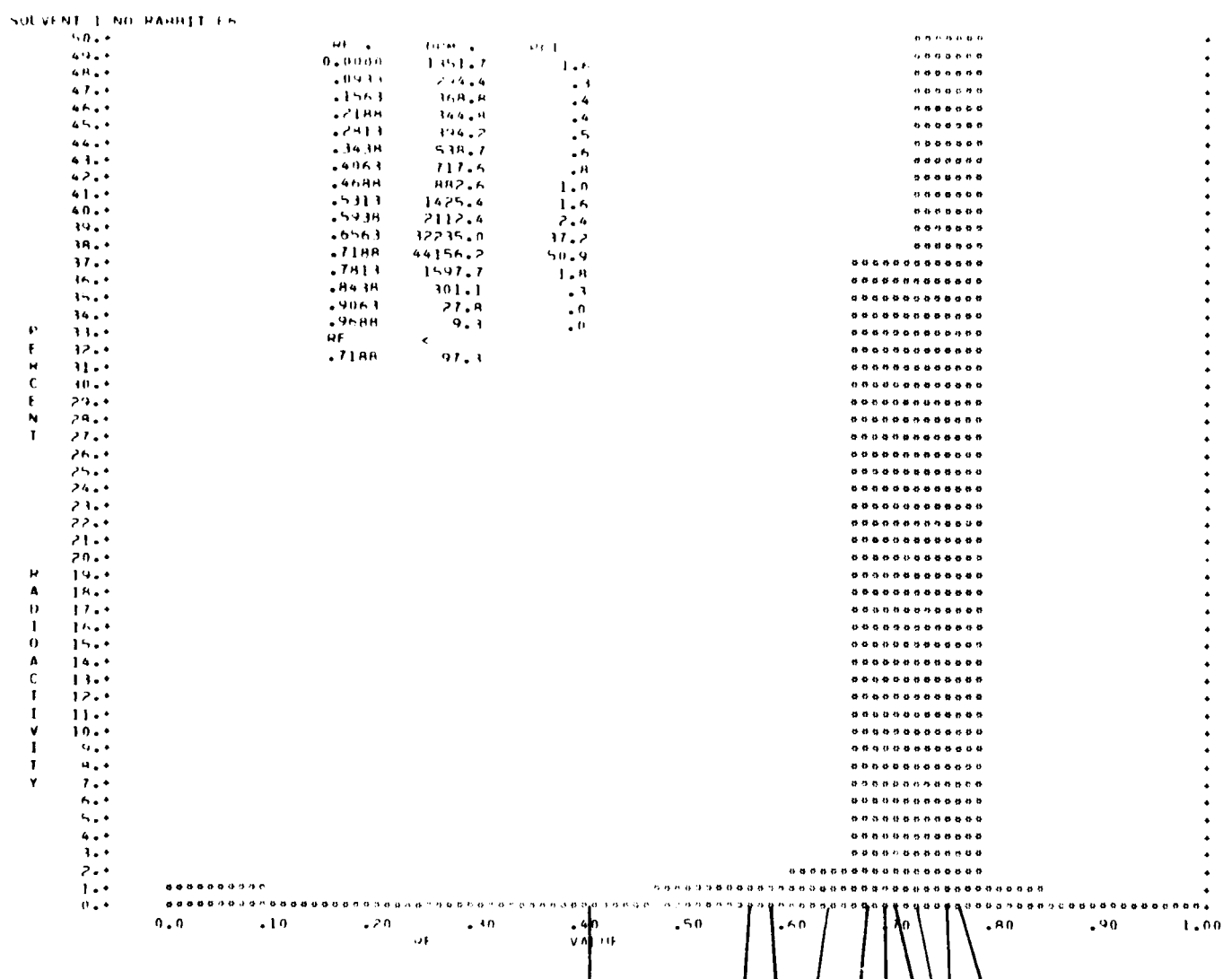

Figure 34-E_6: Solvent I

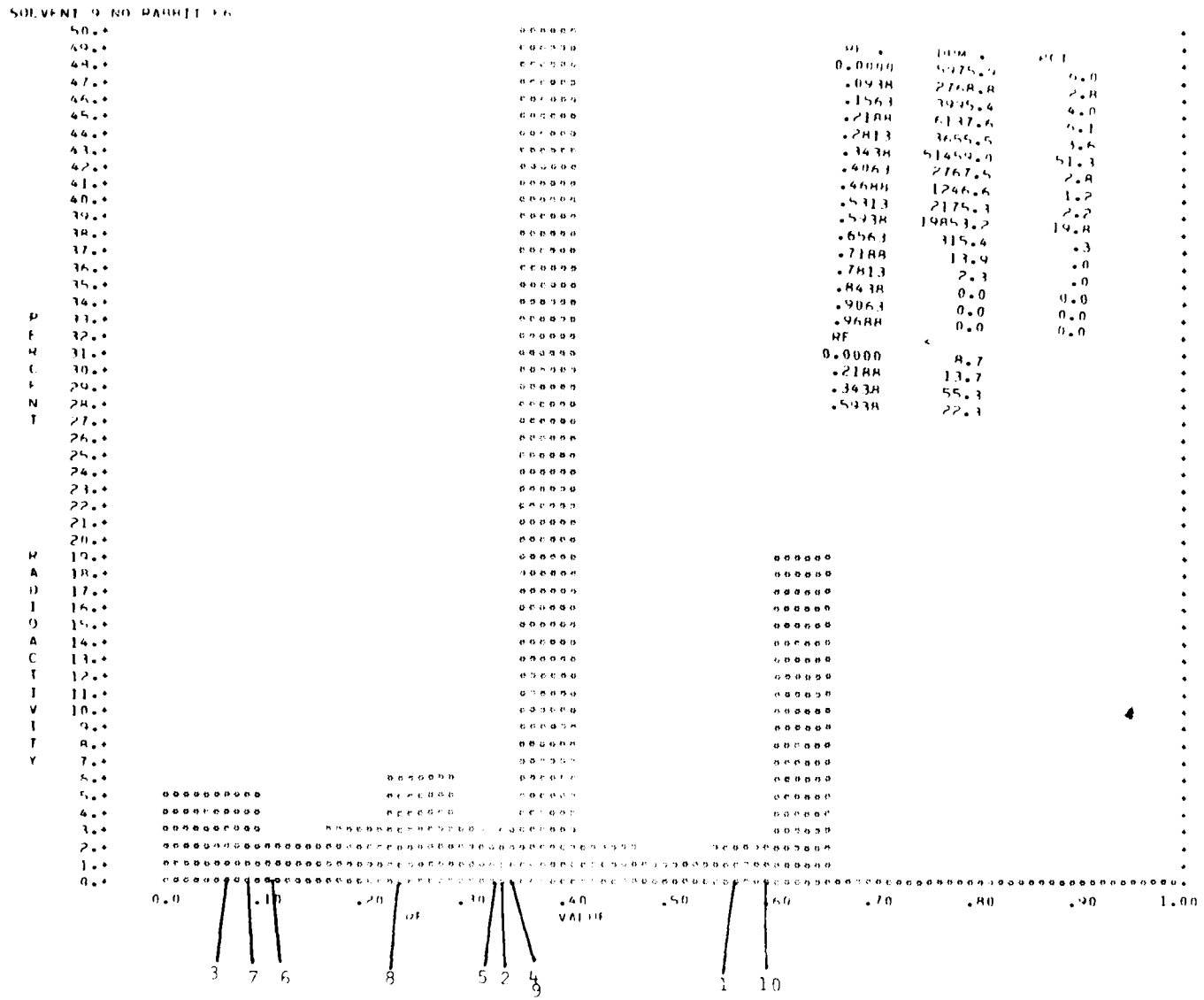

Figure 34-F_6: Solvent IX

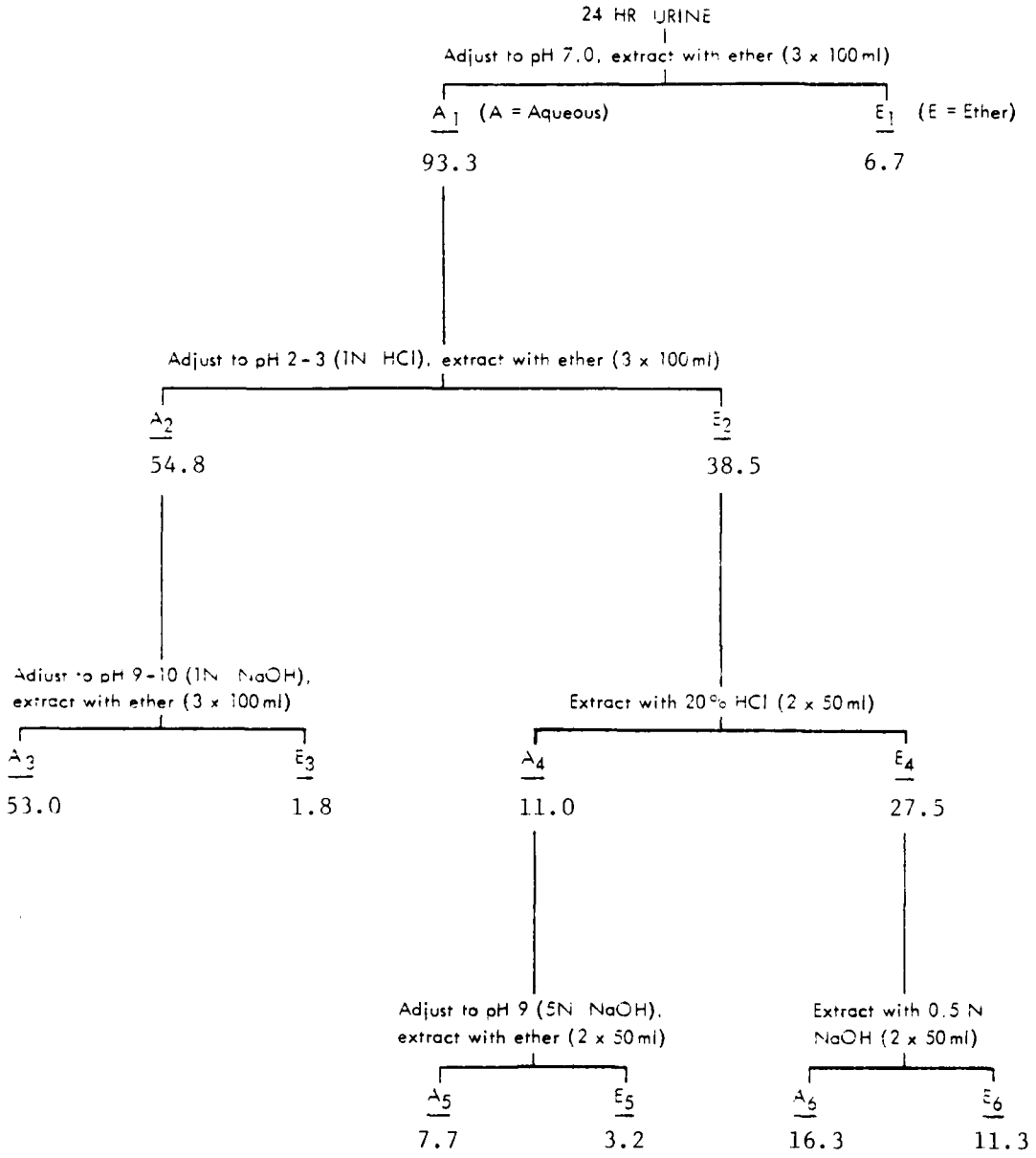

Figure 35: Fractionation of 24-Hr Urine Obtained from Rabbits Treated Dermally with ^{14}C-TNT. Values indicate the percentage of extractable radioactivity in each fraction.

Figure 36, E_1-E_6: TLC of Ether-Extractable Products Obtained from 24-Hr Urine of Rabbits Treated Dermally with ^{14}C-TNT. Extractions were performed at different pH conditions according to the scheme described in the preceding figure. Solvent I, n-butanol:acetic acid: water, 10:1:1; Solvent IX, toluene:acetic acid, 4:1. For reference metabolites (1-10) see Figure 26 or Table 19. Reference metabolites are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzyl alcohol
3. Trinitrobenzoic Acid
4. 4-Amino-2,6-dinitrotoluene
5. 2-Amino-4,6-dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2',6'-Tetranitro-4,4'-azoxytoluene

Figure 36 follows

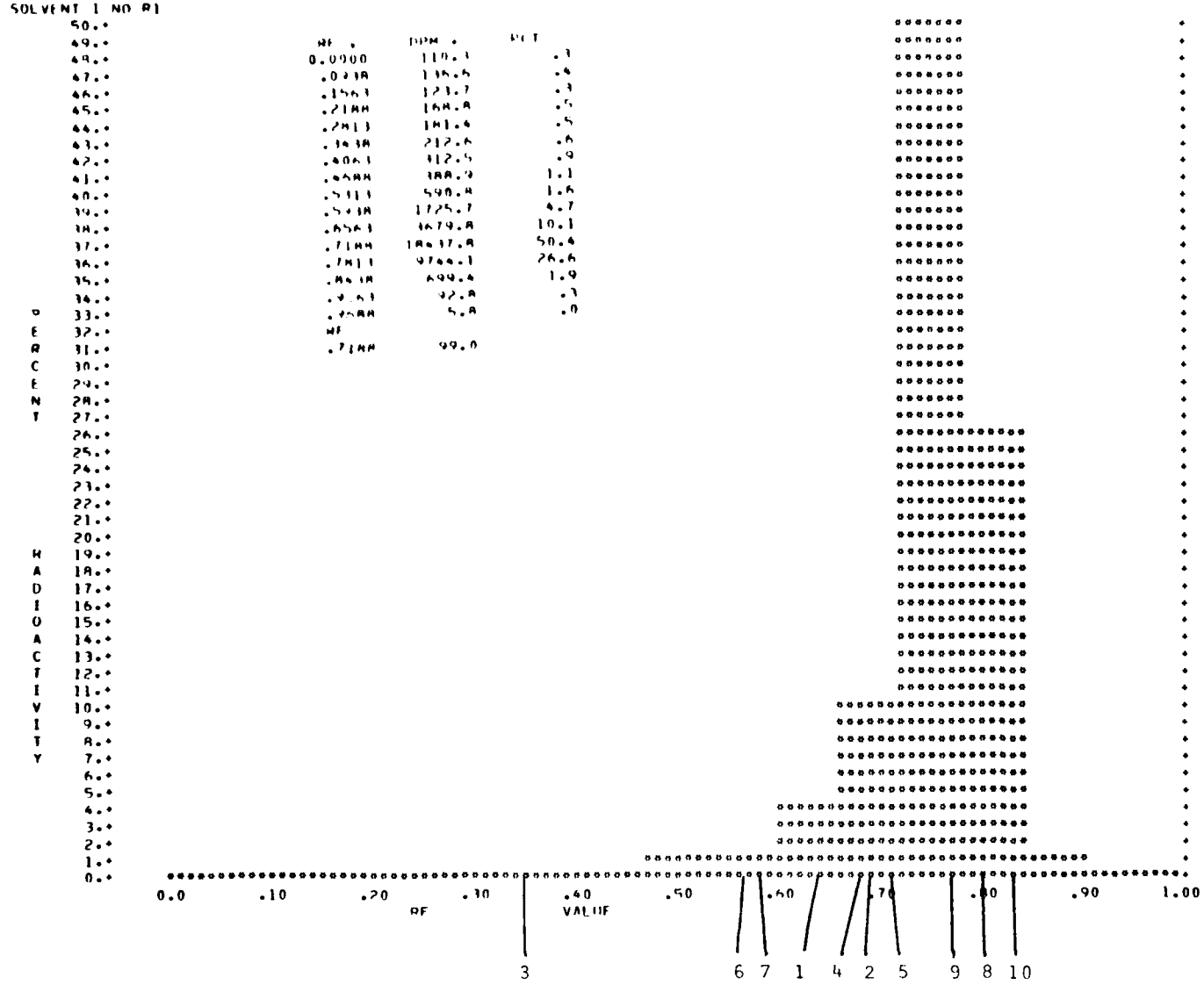

Figure 36-E_1: **Solvent I.**

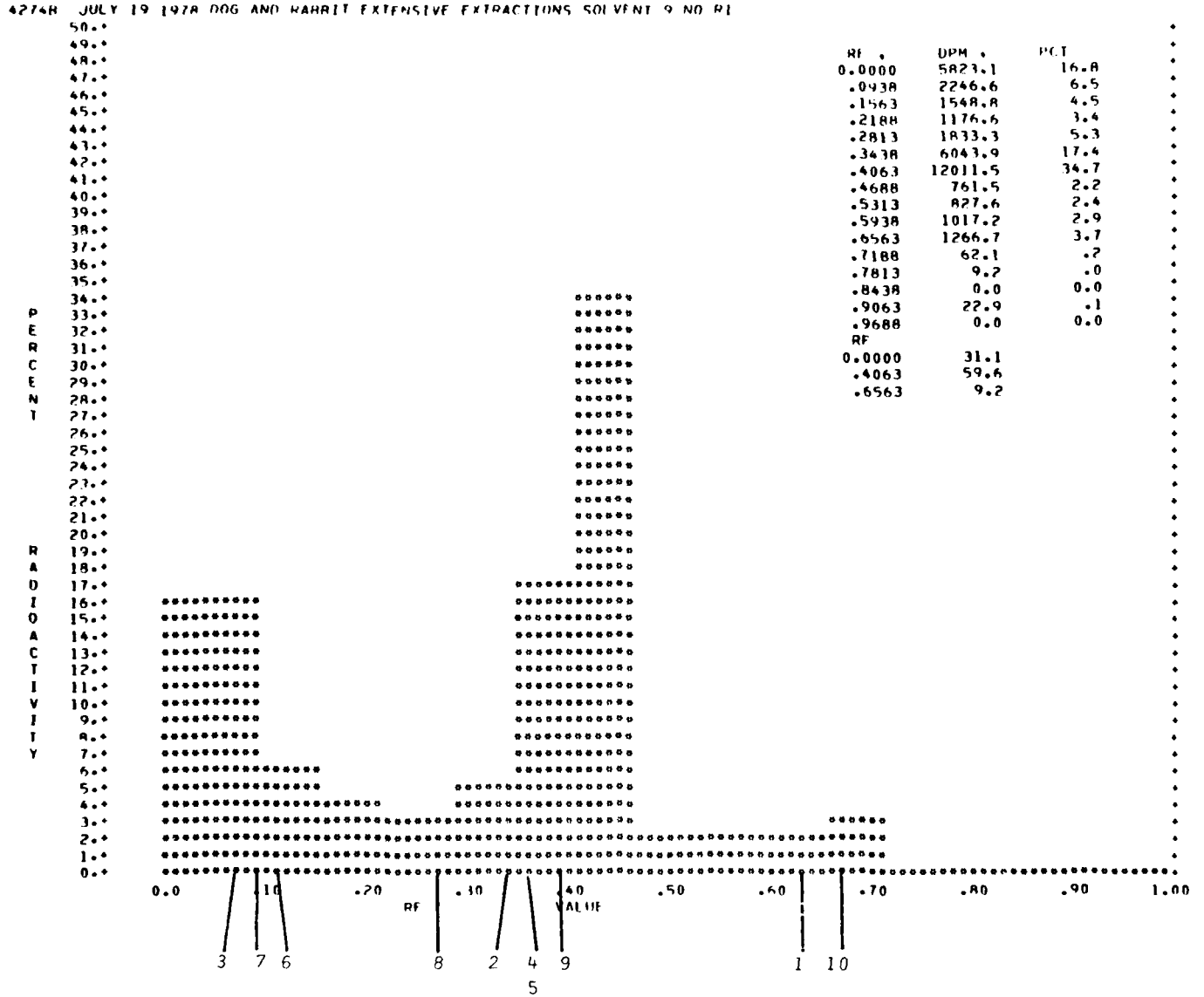

Figure 36-E_1: **Solvent IX.**

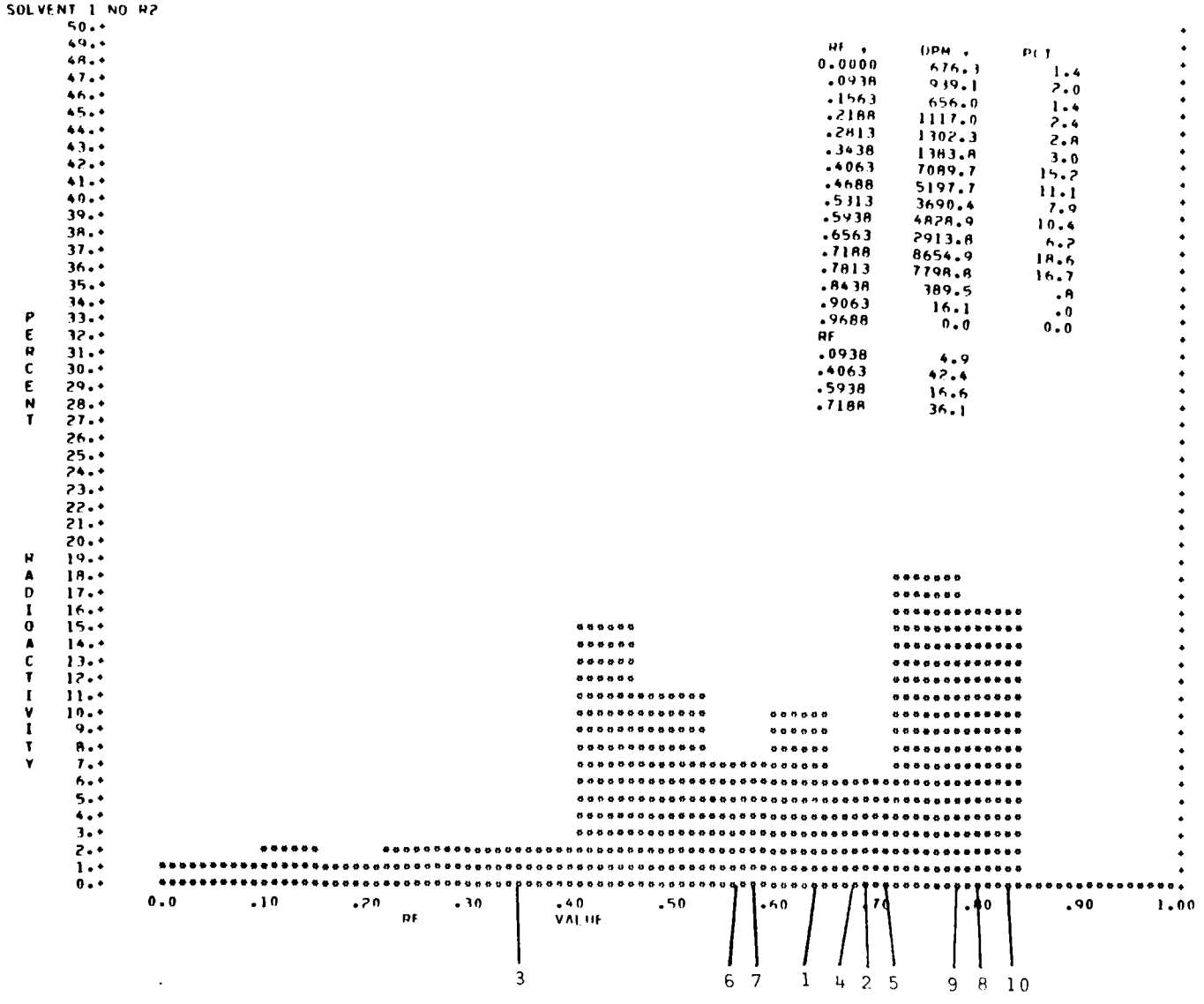

Figure 36-E_2: **Solvent I.**

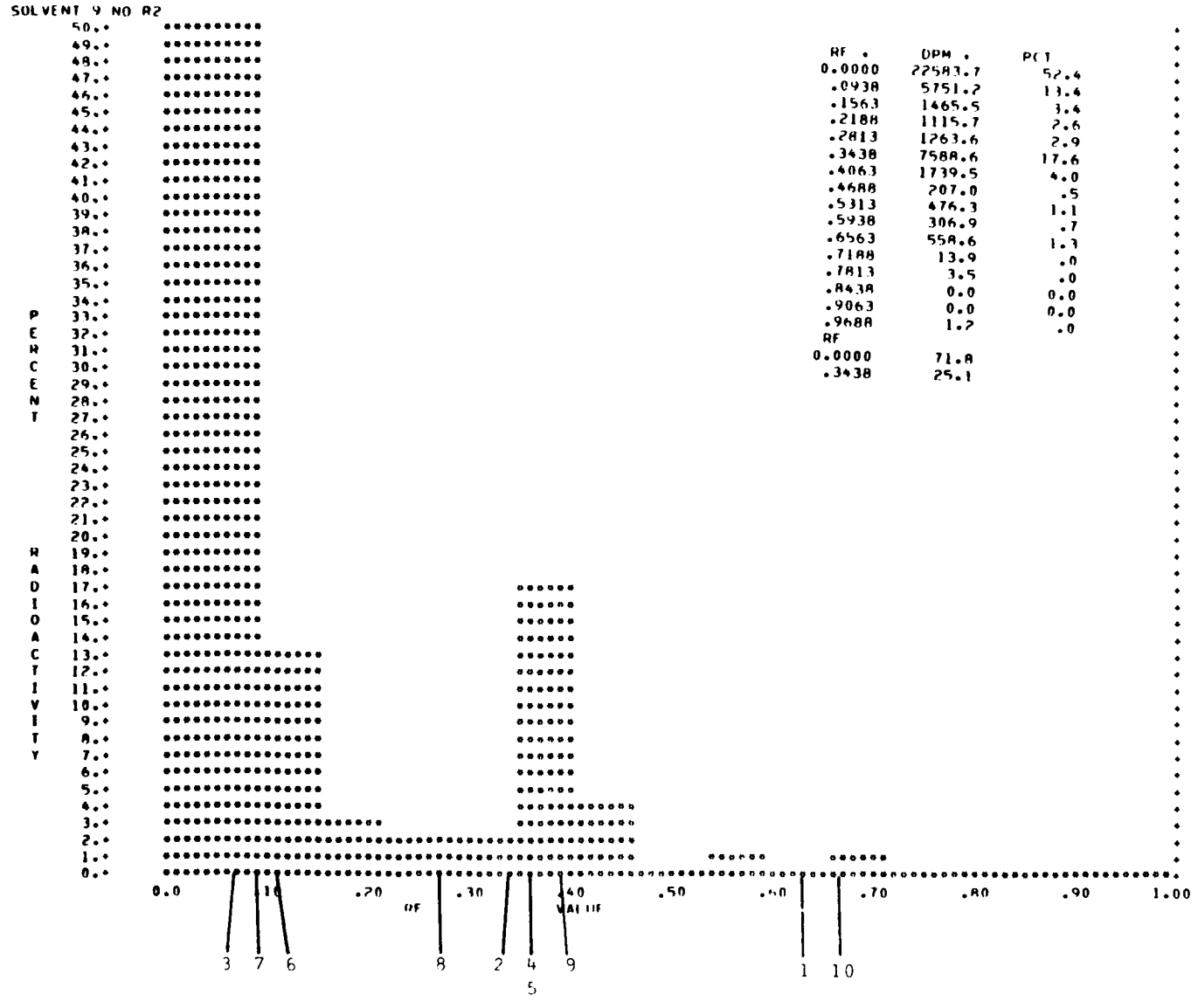

Figure 36-E_2: Solvent IX.

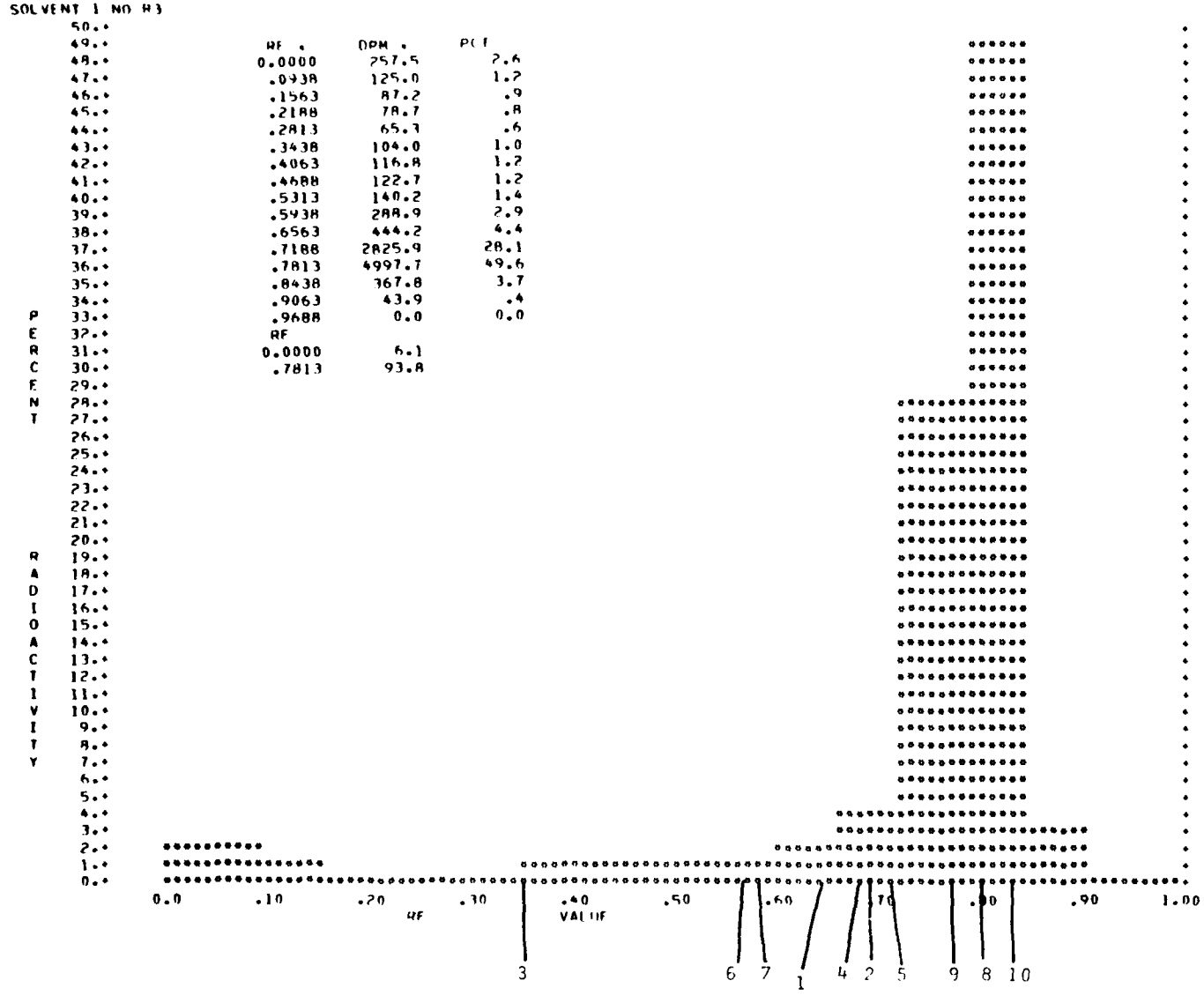

Figure 36-E_3: Solvent I.

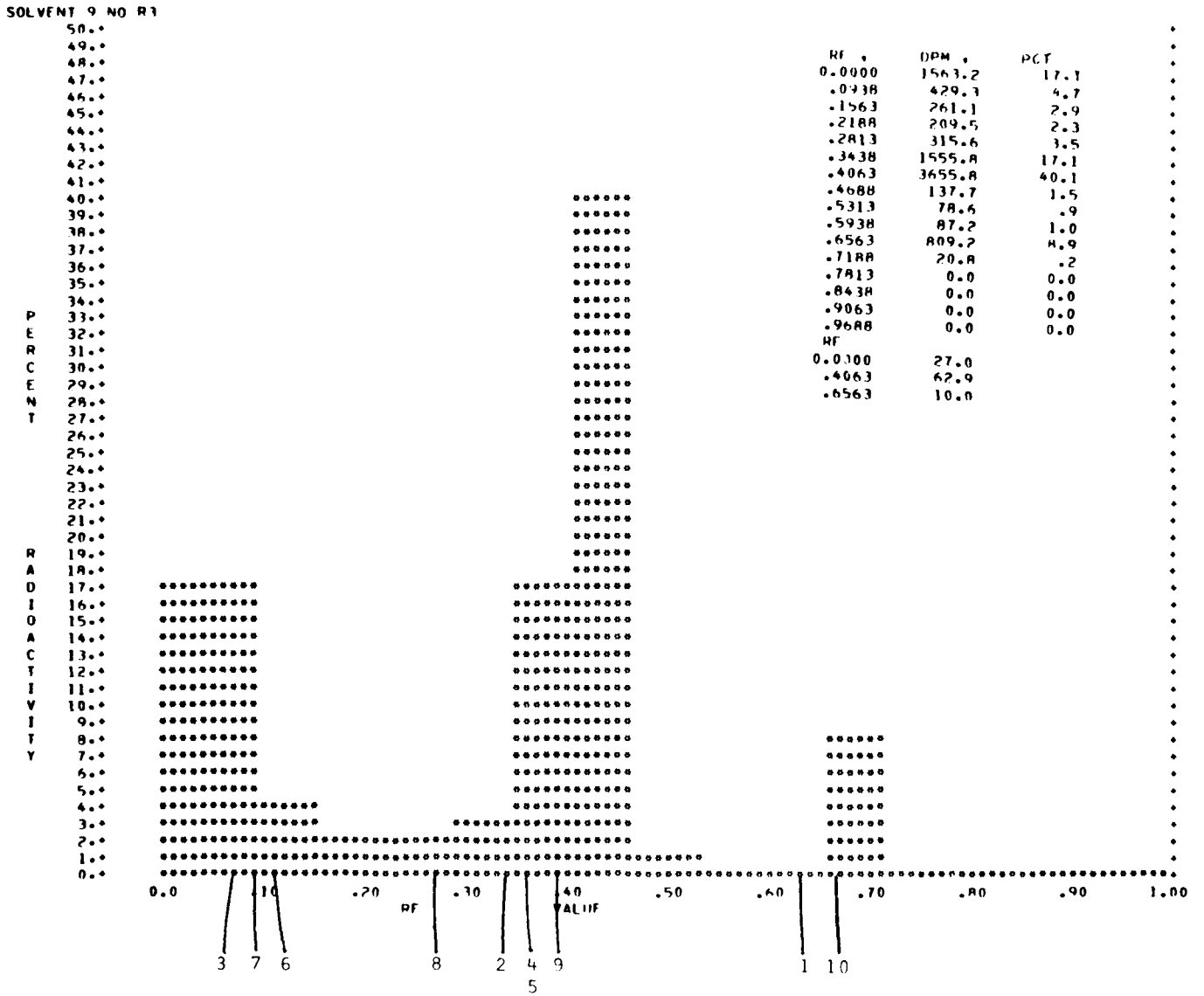

Figure 36-E_3: **Solvent IX.**

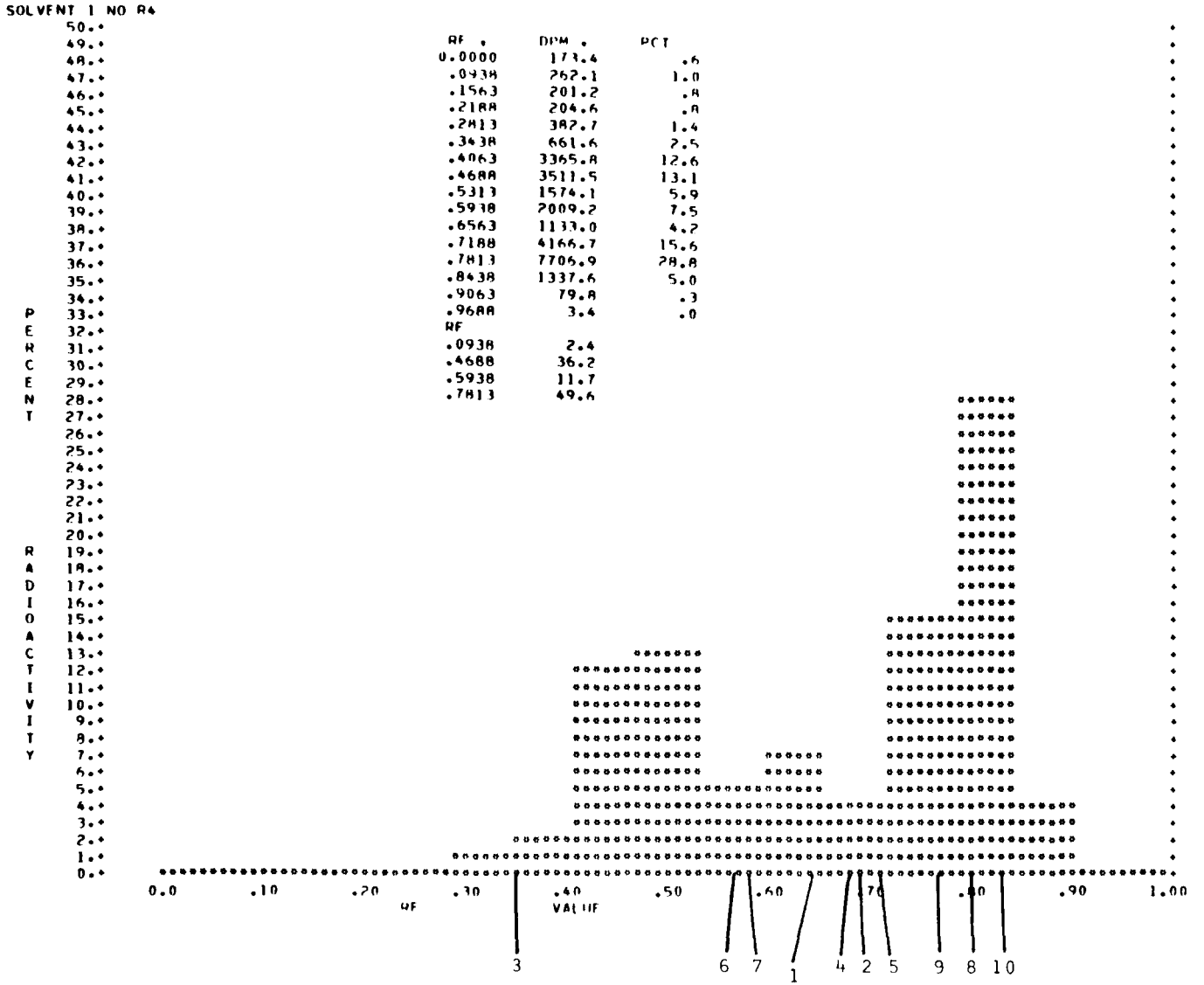

Figure 36-E_4: **Solvent I.**

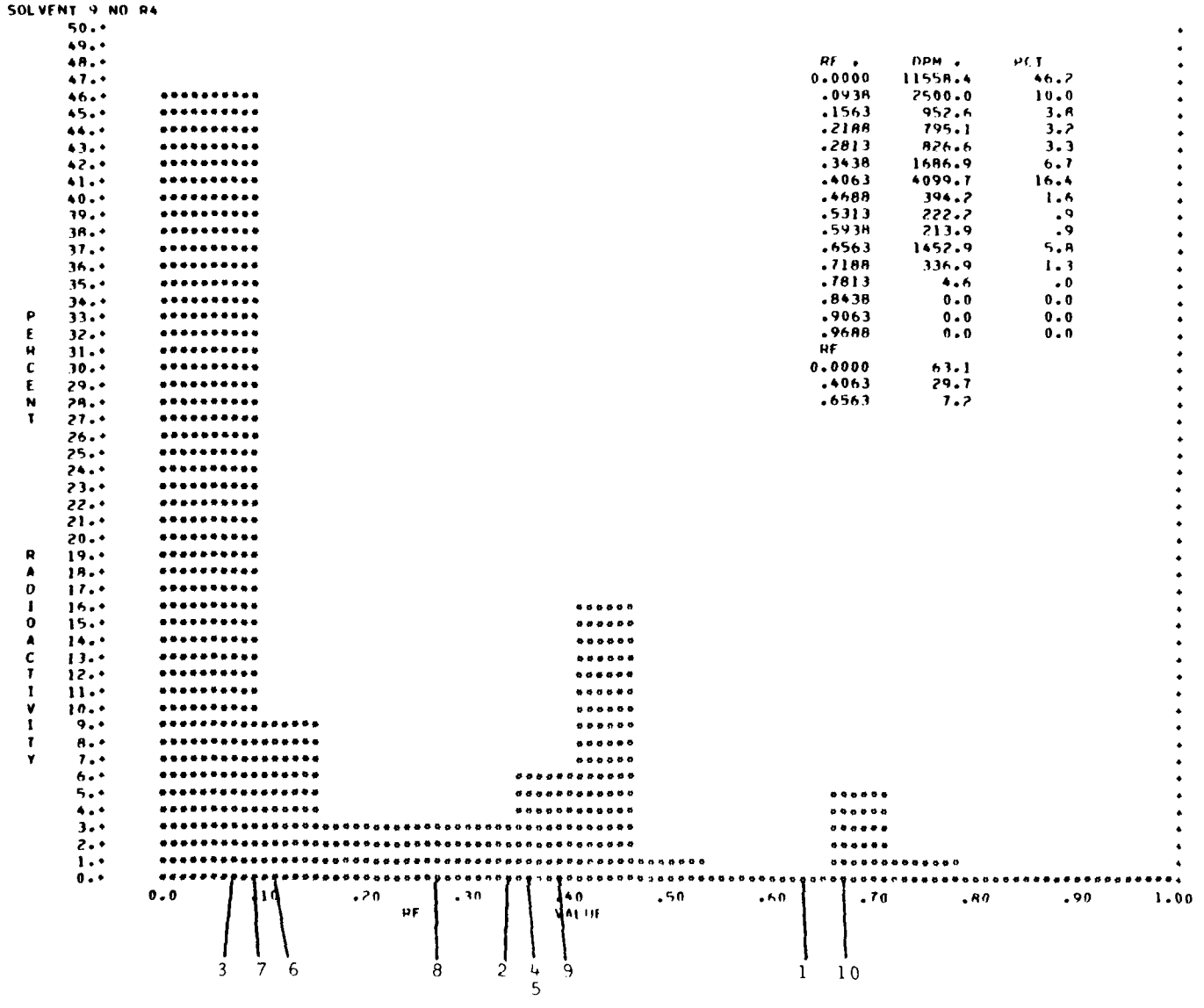

Figure 36-E_4: Solvent IX.

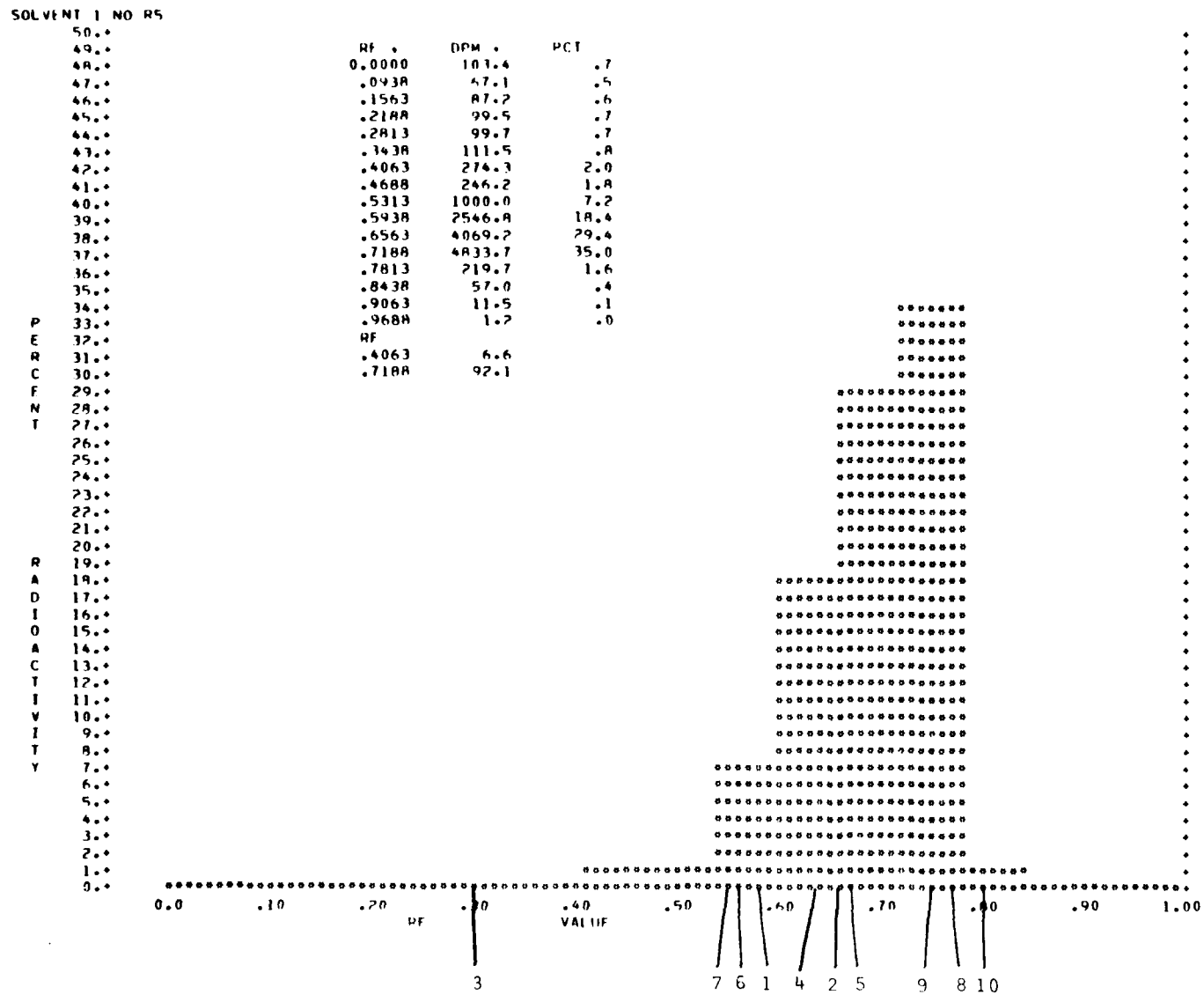

Figure 36-E_5: **Solvent I.**

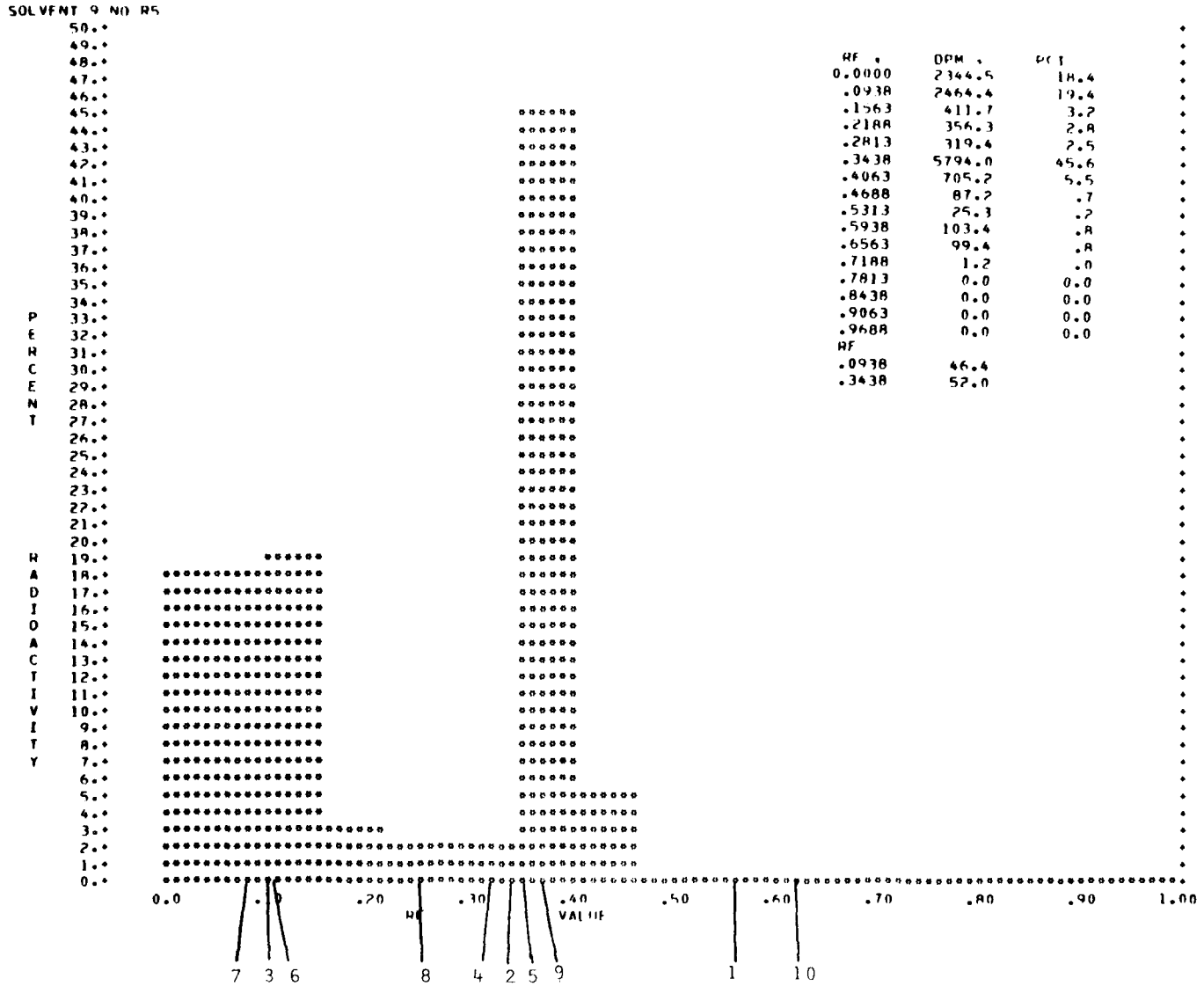

Figure 36-E_5: **Solvent IX.**

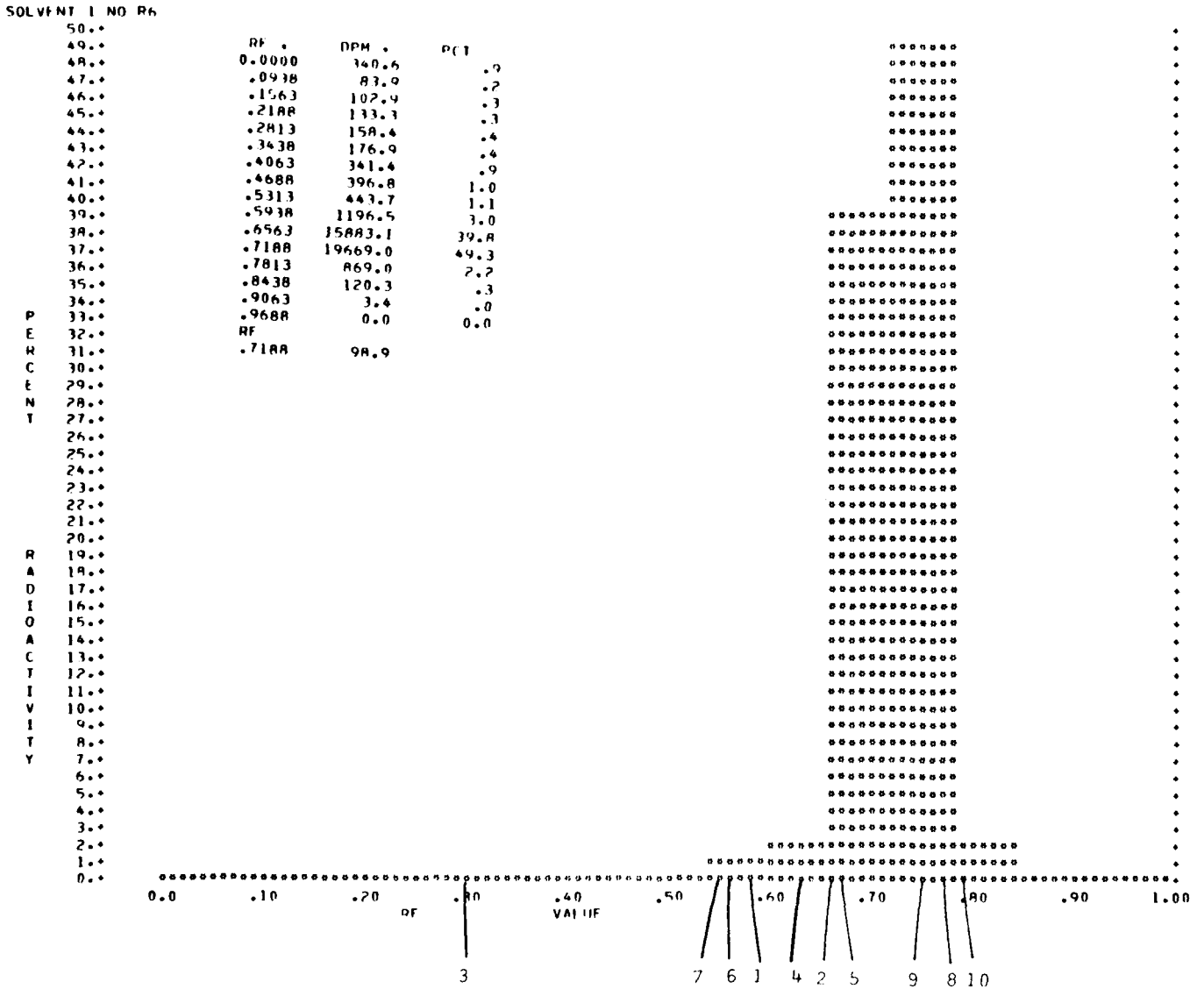

Figure 36-E_6: **Solvent I.**

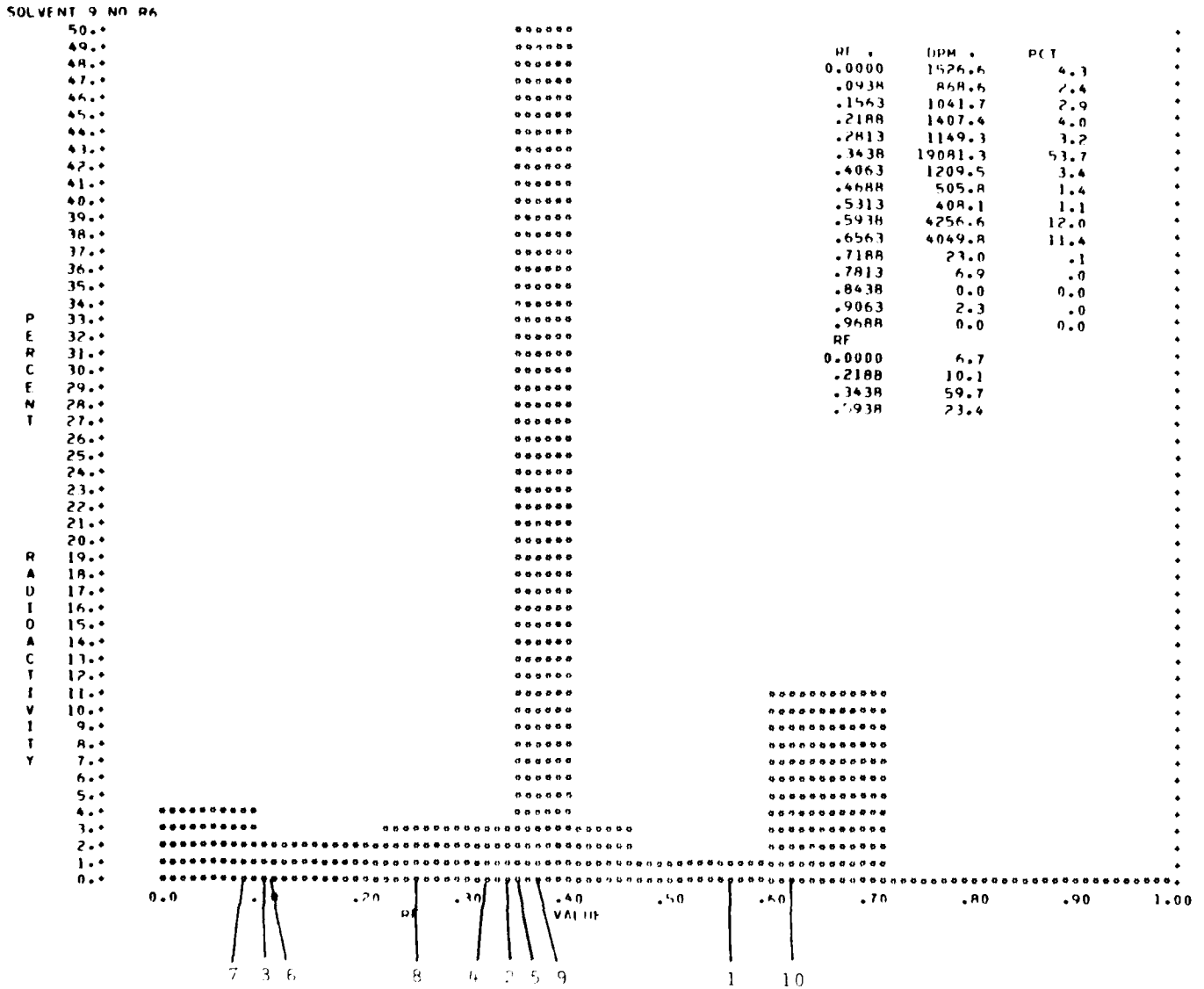

Figure 36-E_6: **Solvent IX.**

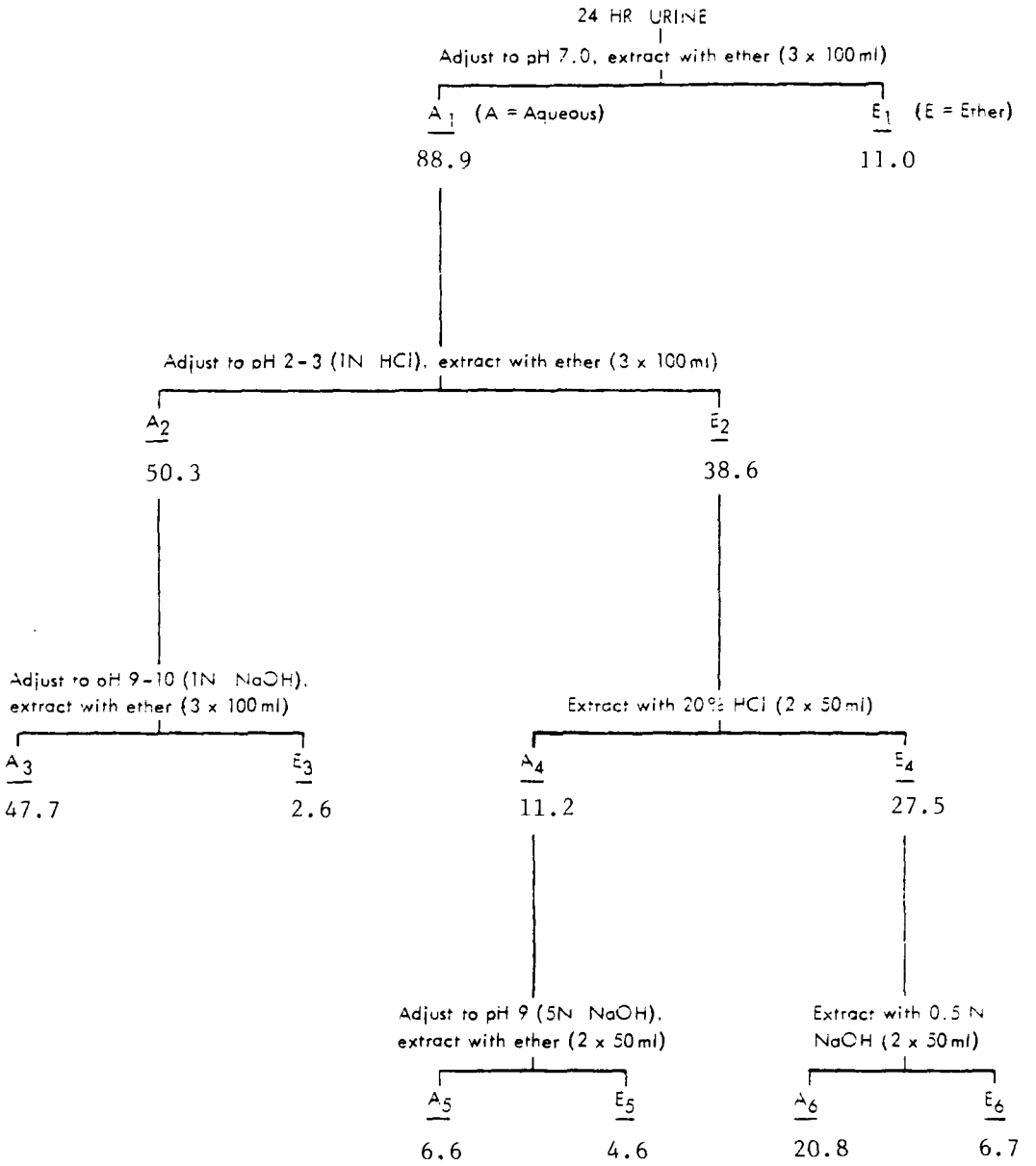

Figure 37: Fractionation of 24-Hr Urine Obtained from Dogs Treated Orally with ^{14}C-TNT. Values indicate the percentage of extractable radioactivity in each fraction.

Figure 38, E_1-E_6: TLC of Ether-Extractable Products Obtained from 24-Hr Urine of Dogs Treated Orally with ^{14}C-TNT. Extractions were performed at different pH conditions according to the scheme described in the preceding figure. Solvent I, n-butanol:acetic acid:water, 10:1:1; Solvent IX, toluene:acetic acid, 4:1. For reference metabolites (1-10) see Figure 26 or Table 19. Reference metabolites are:

1. Trinitrotoluene (TNT)
2. Trinitrobenzyl alcohol
3. Trinitrobenzoic Acid
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5. 2-Amino-4,6-dinitrotoluene
6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2',6'-Tetranitro-4,4'-azoxytoluene

Figure 38 follows

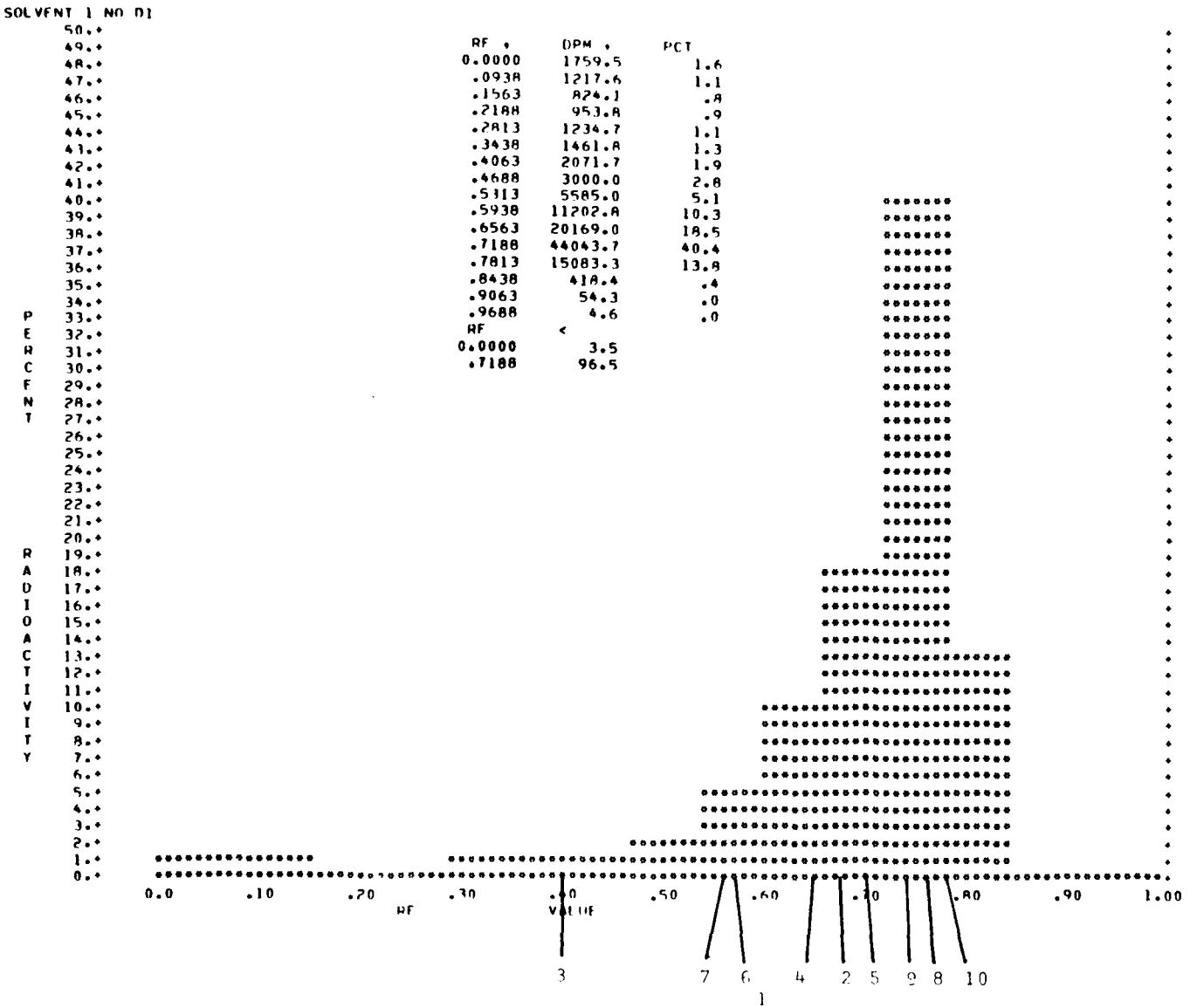

Figure 38-E_1: Solvent I

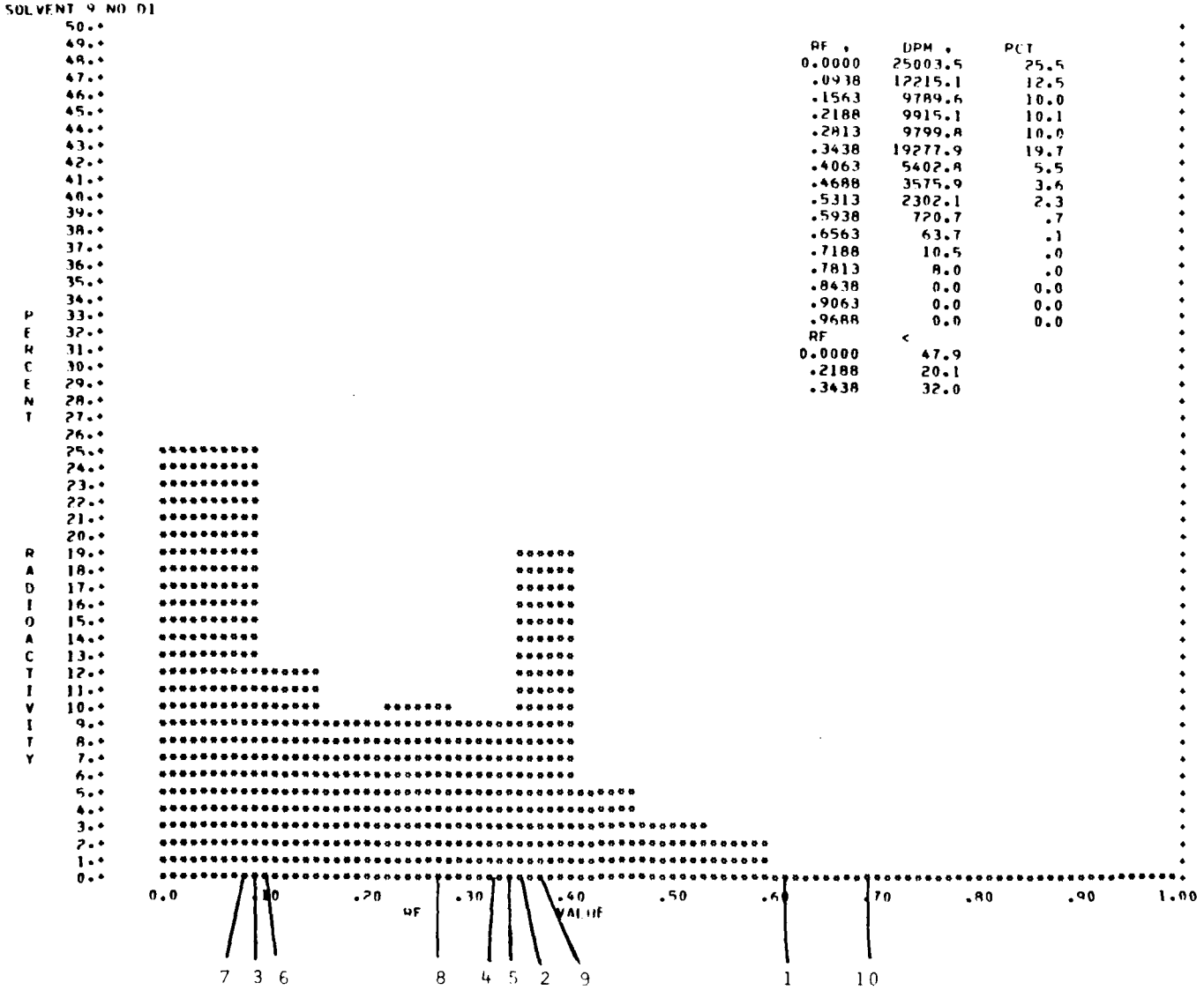

Figure 38-E1: Solvent IX

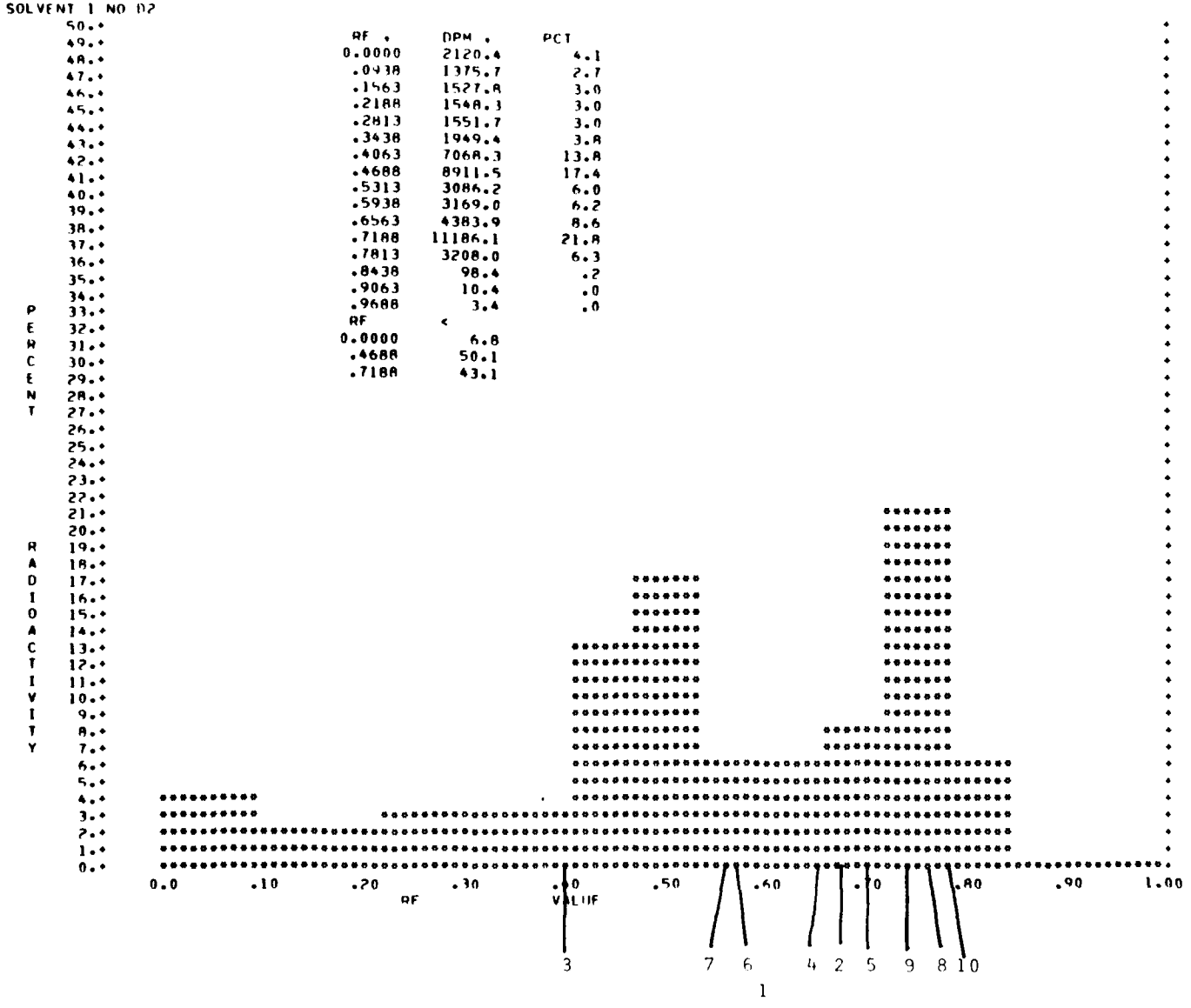

Figure 38-E_2: Solvent I

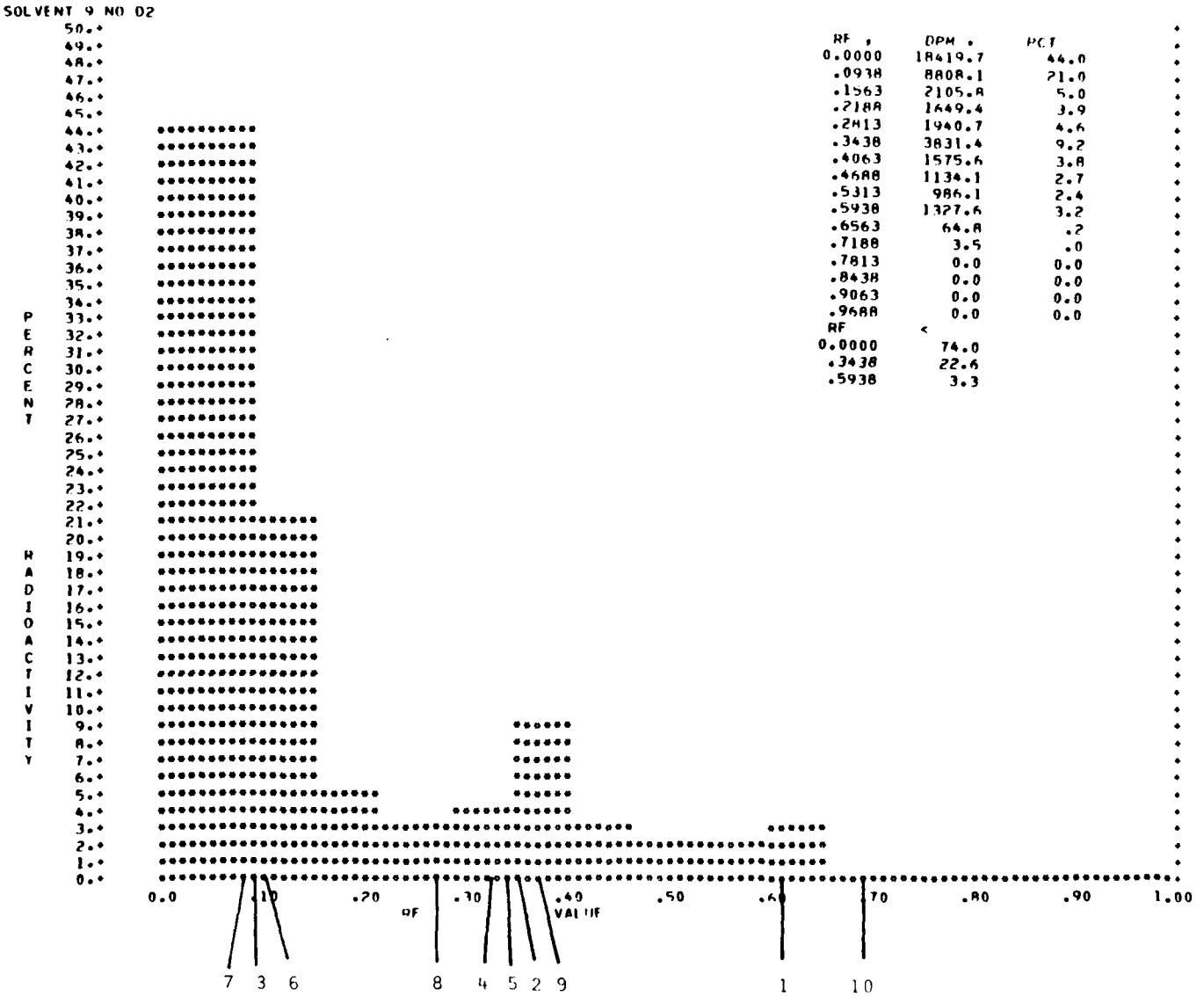

Figure 38-E_2: Solvent IX

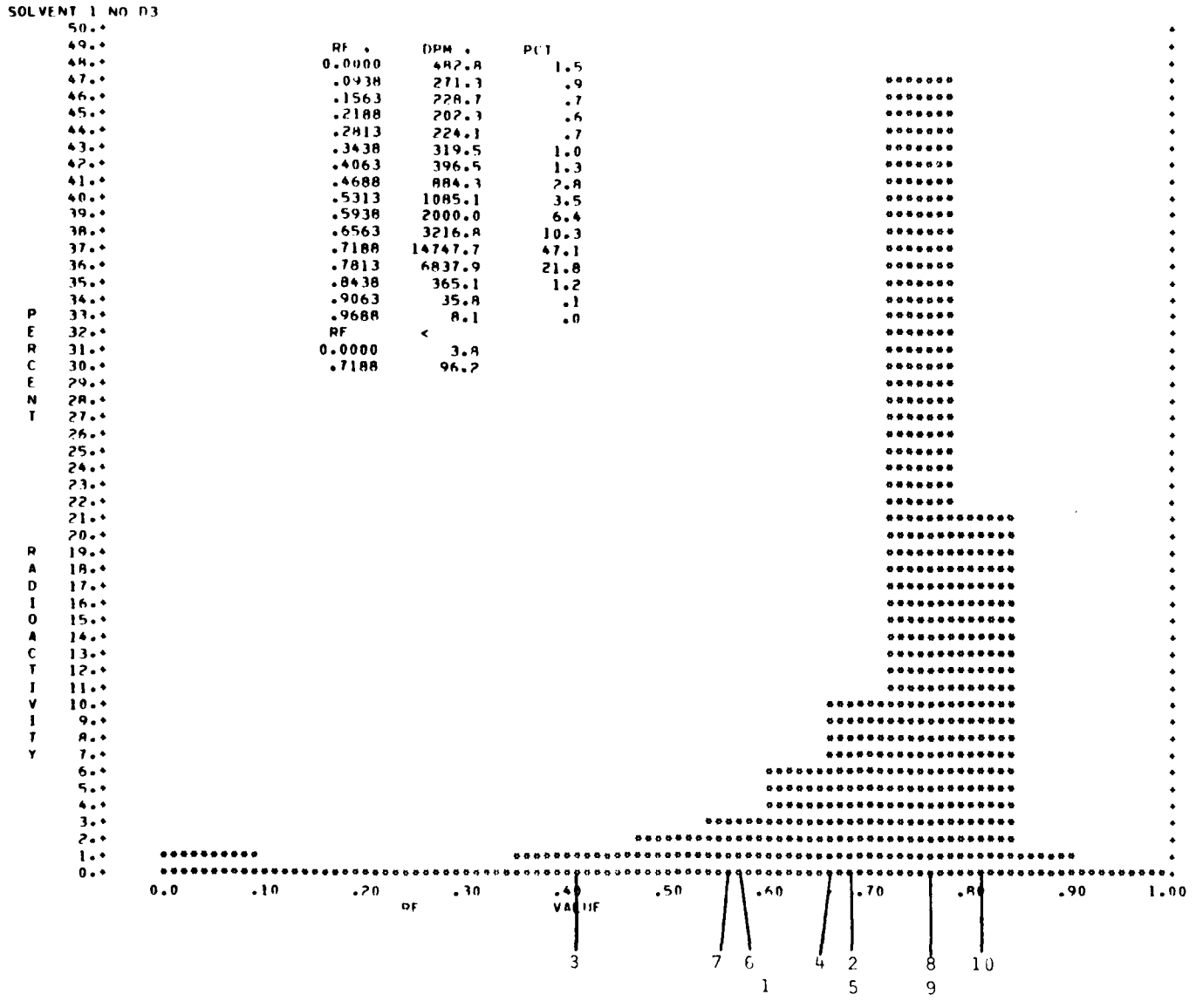

Figure 38-E_3: Solvent I

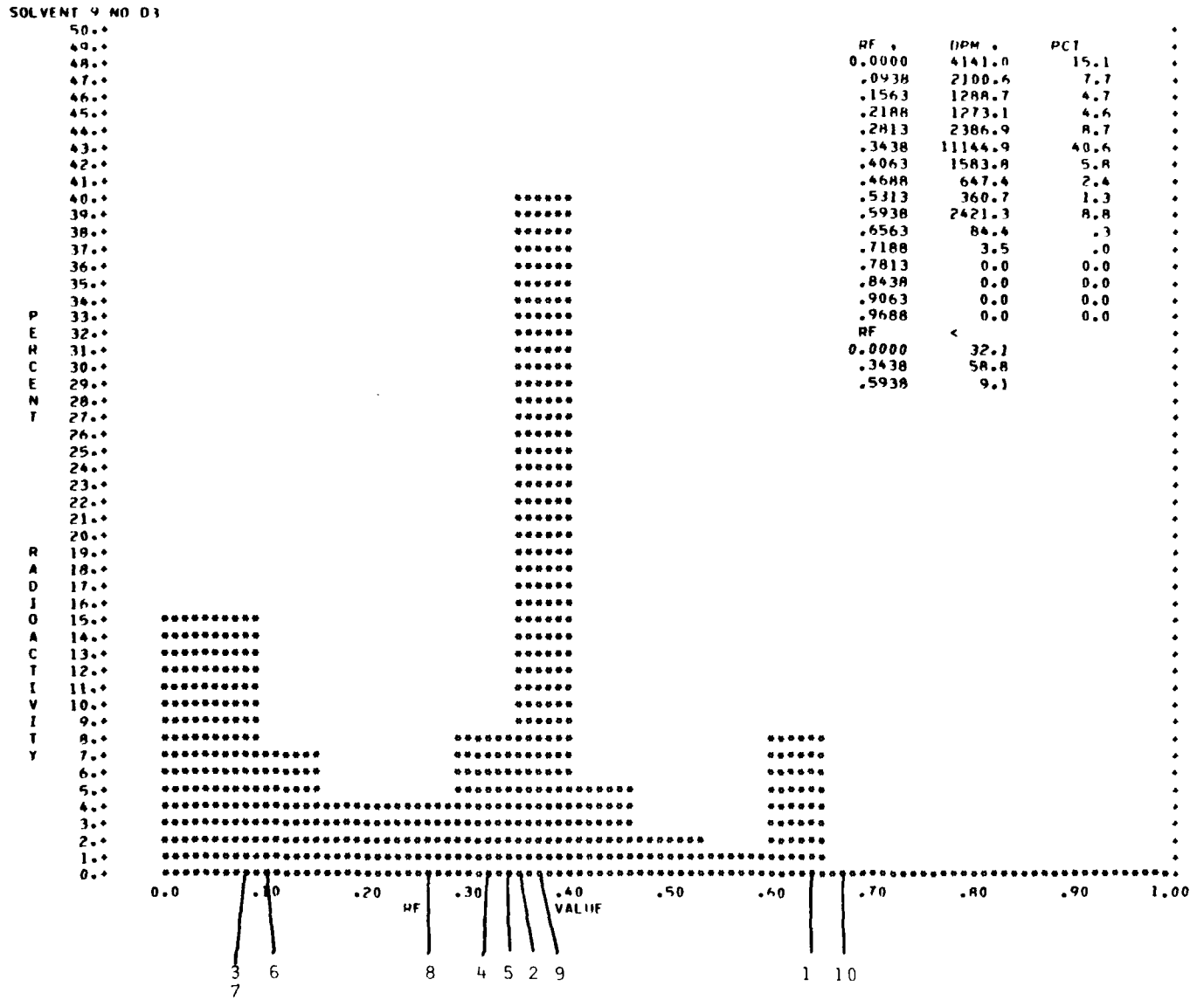

Figure 38-E_3: Solvent IX

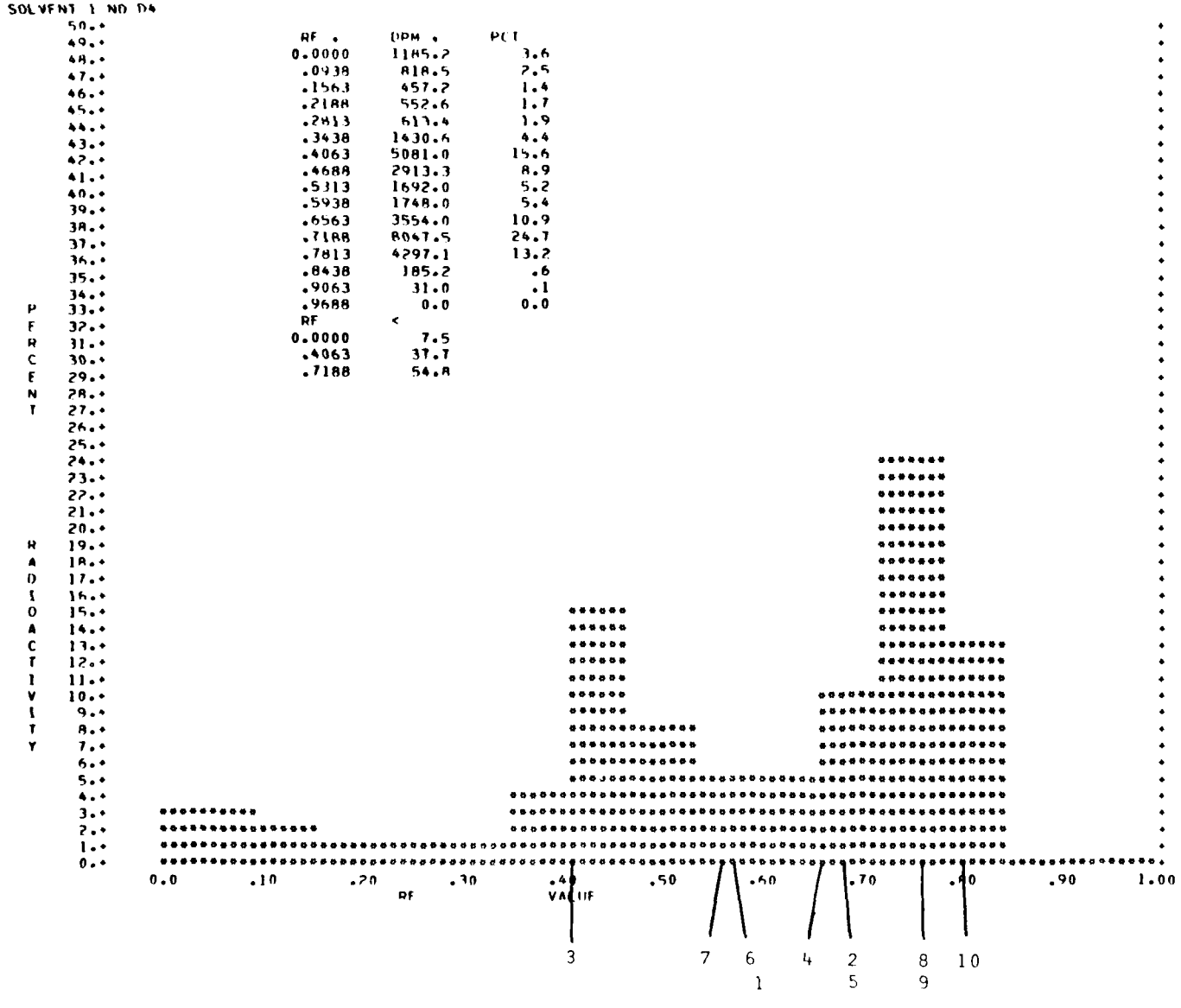

Figure 38-E_4: Solvent I

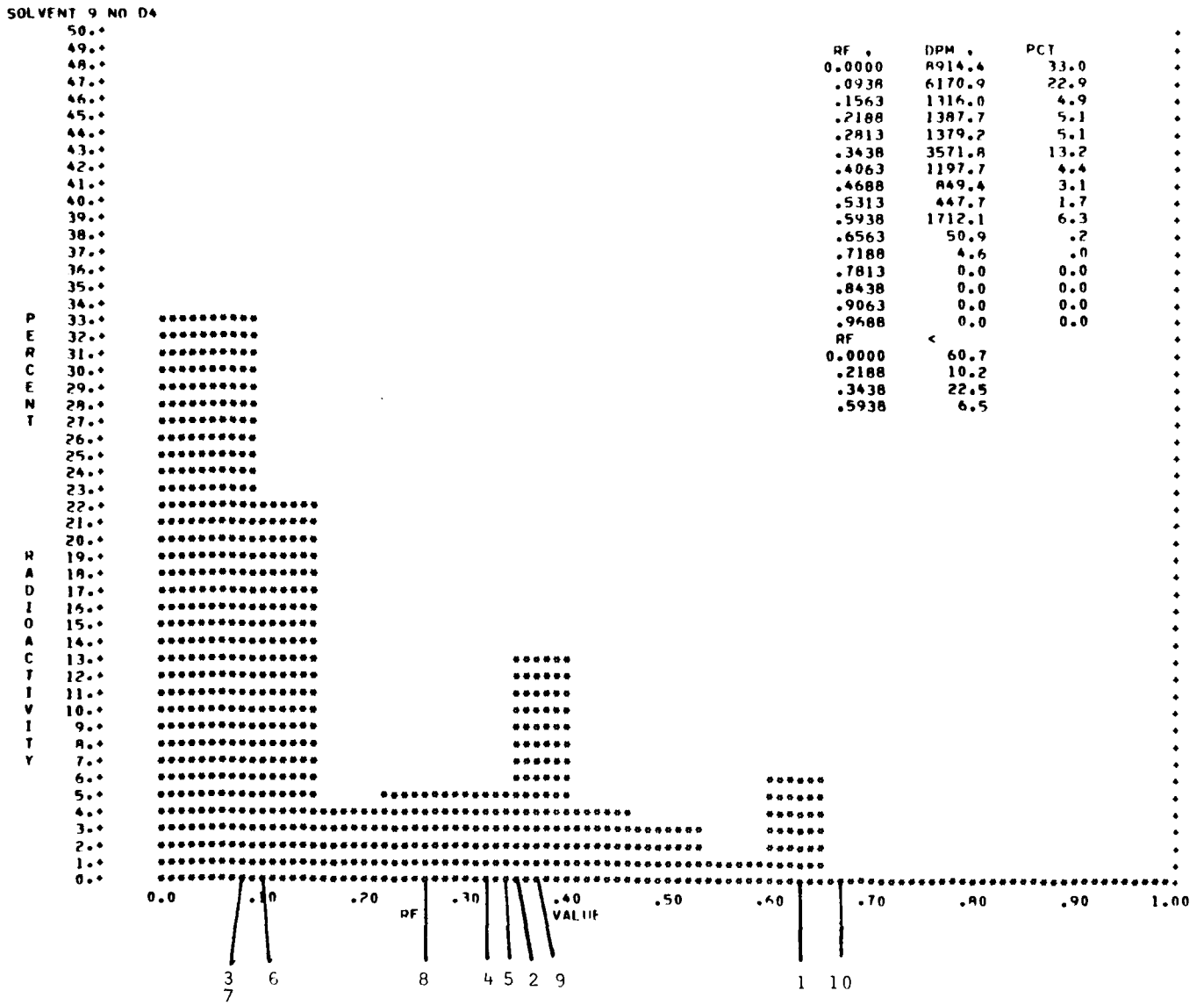

Figure 38-E4: Solvent IX

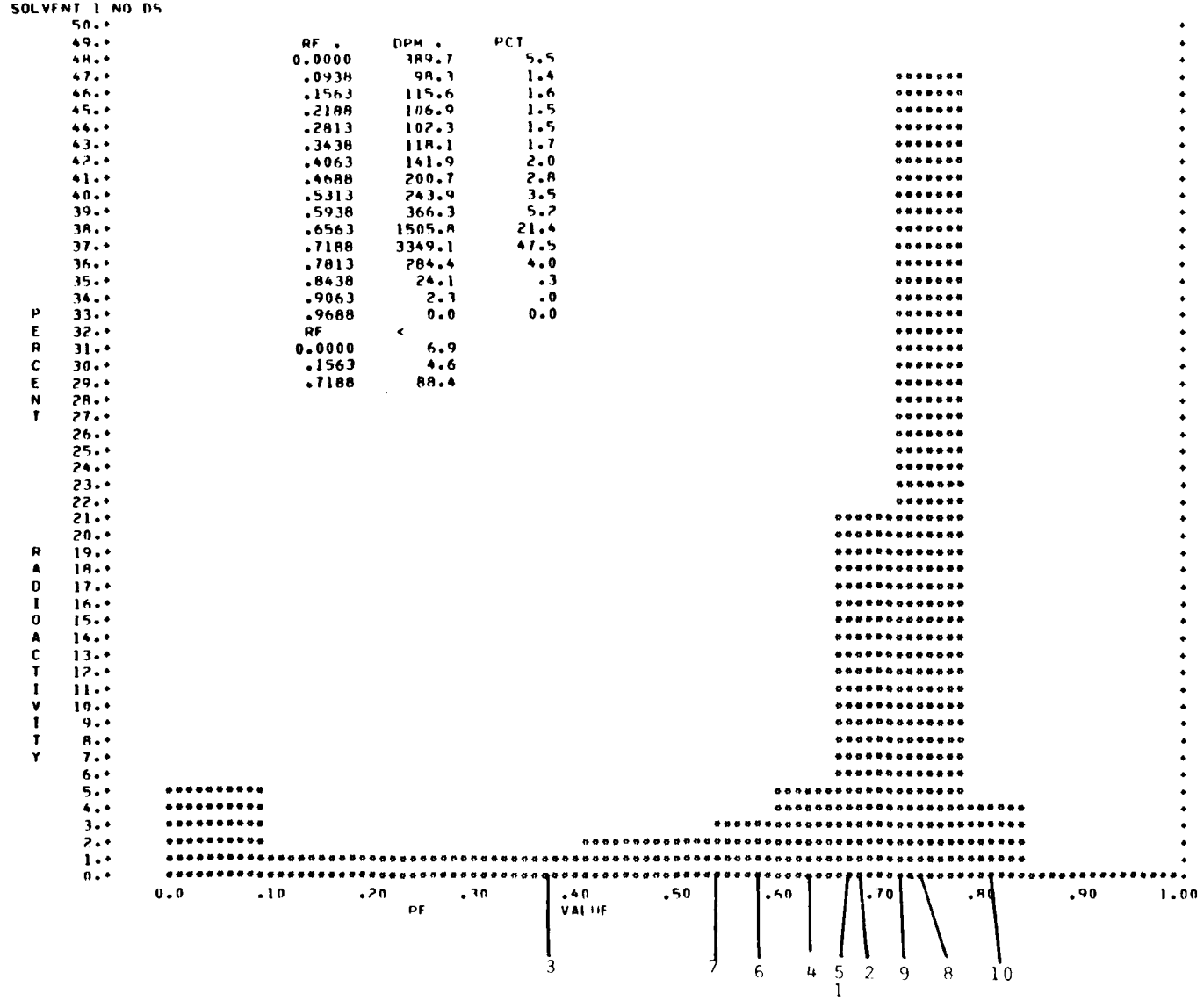

Figure 38-E_5: Solvent I

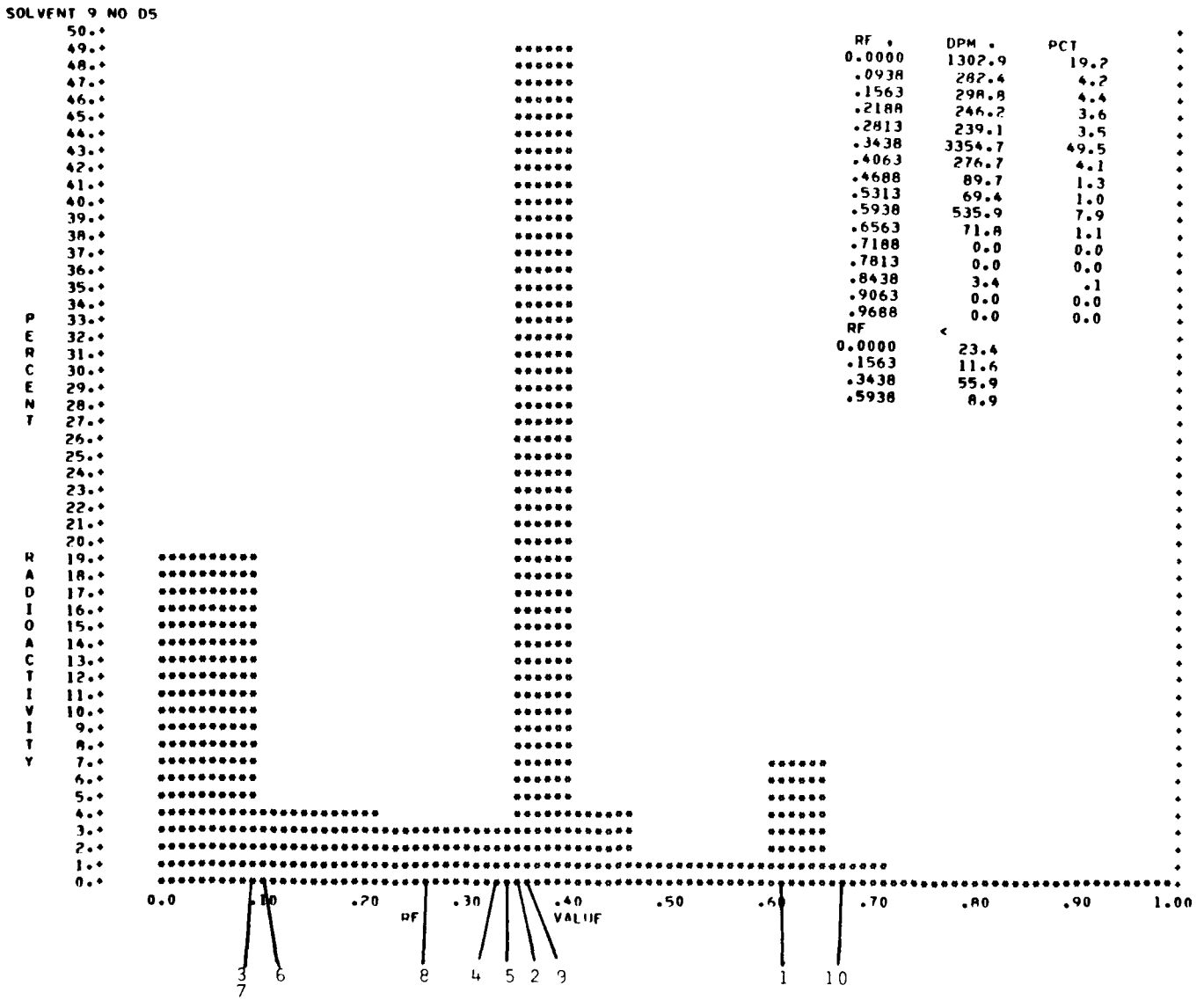

Figure 38-E_5: Solvent IX

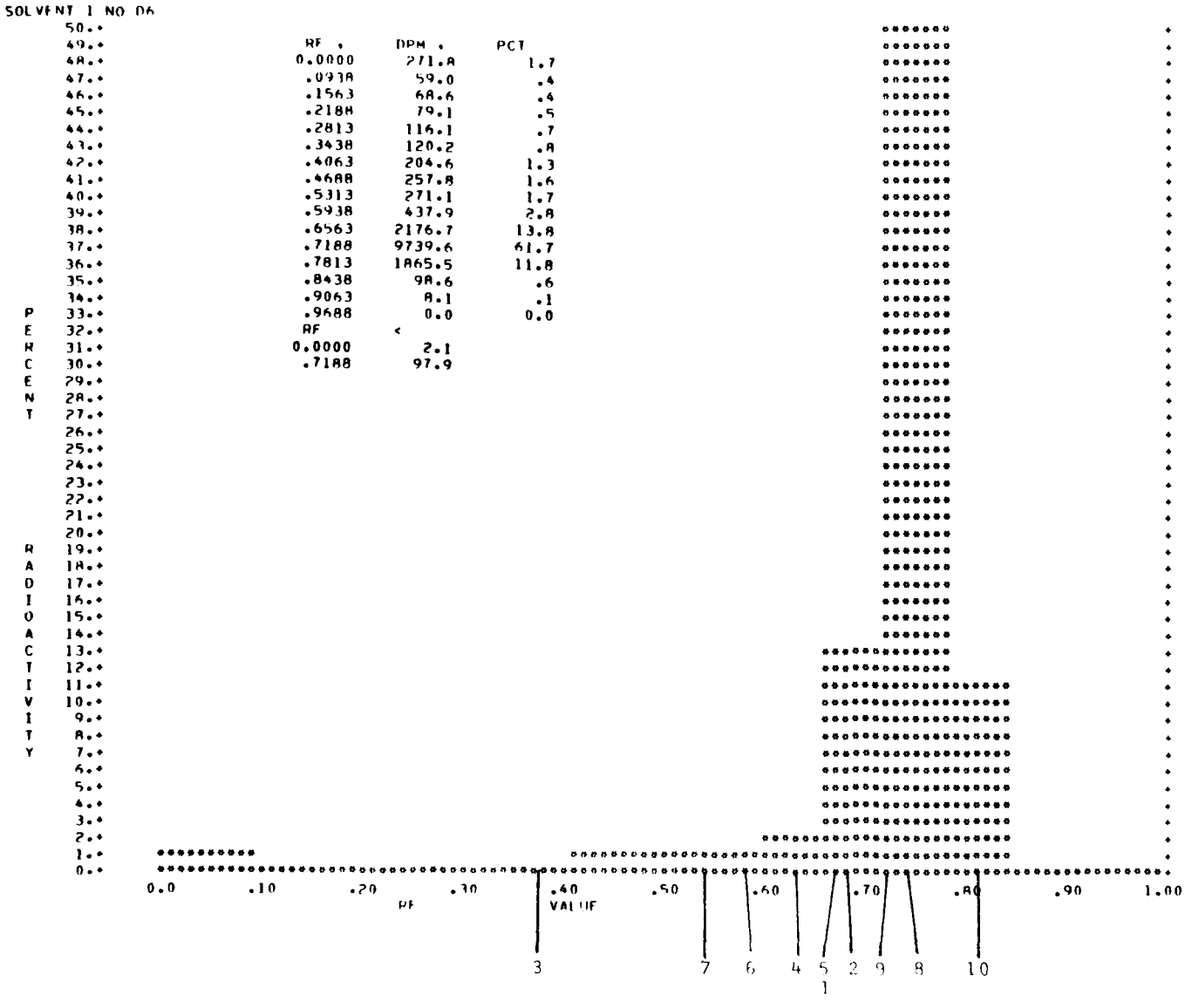

Figure 38-E_6: Solvent I

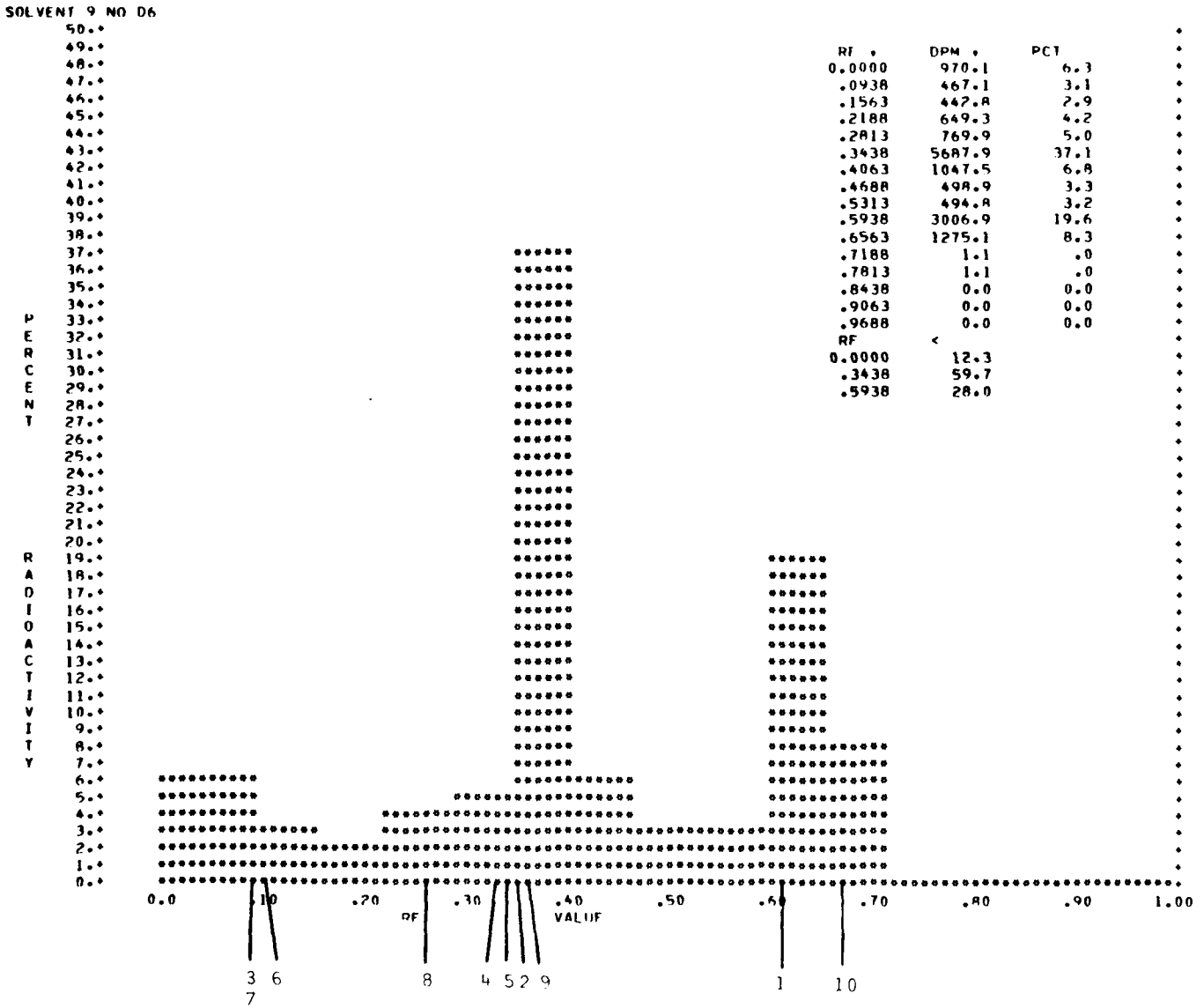

Figure 38-E_6: Solvent IX

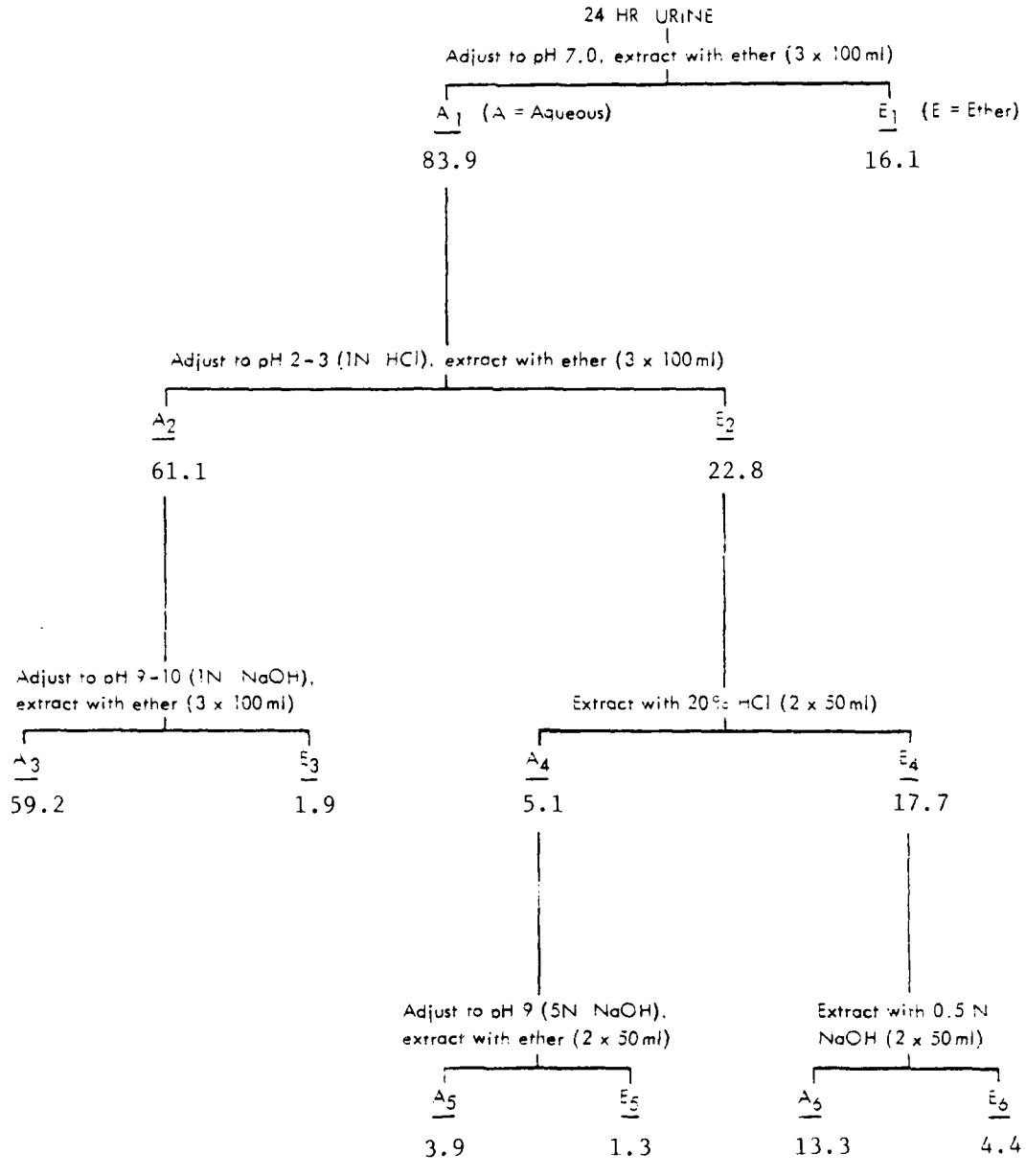

Figure 39: Fractionation of 24-Hr Urine from Dogs Treated Dermally with ^{14}C-TNT. Values indicate the percentage of extractable radioactivity in each fraction.

Figure 40, E_1-E_6: TLC of Ether-Extractable Products Obtained from 24-Hr Urine of Dogs Treated Dermally with ^{14}C-TNT. Extractions were performed at different pH conditions according to the scheme described in the preceding figure. Solvent I, n-butanol:acetic acid:water, 10:1:1; Solvent IX, toluene:acetic acid, 4:1. For reference metabolites (1-10) see Figure 26 or Table 19. Reference metabolites are:

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6. 4,6-Diamino-2-nitrotoluene
7. 2,6-Diamino-4-nitrotoluene
8. 4-Hydroxylamino-2,6-dinitrotoluene
9. 2-Hydroxylamino-4,6-dinitrotoluene
10. 2,6,2',6'-Tetranitro-4,4'-azoxytoluene

Figure 40 follows

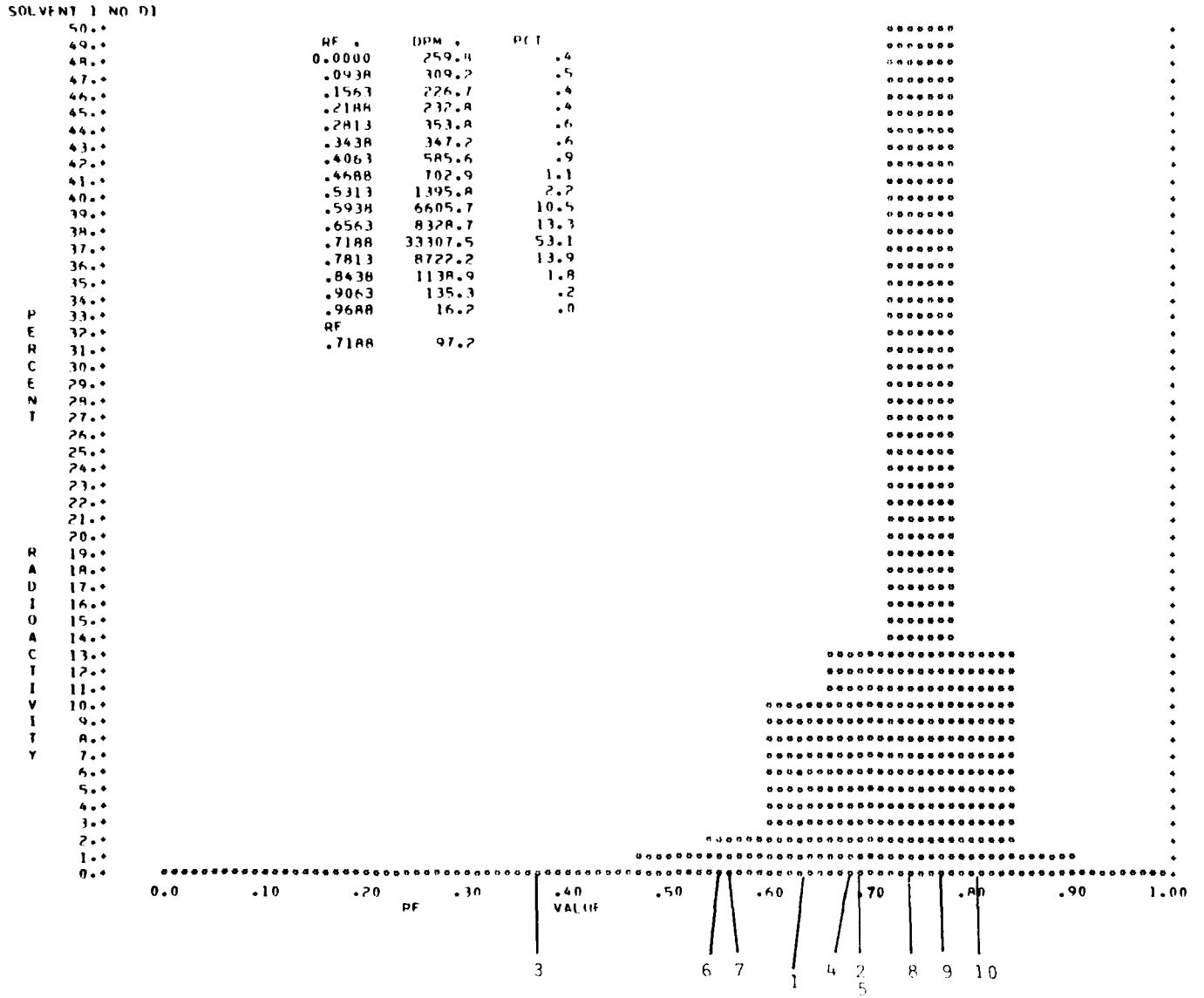

Figure 40-E_1: Solvent I

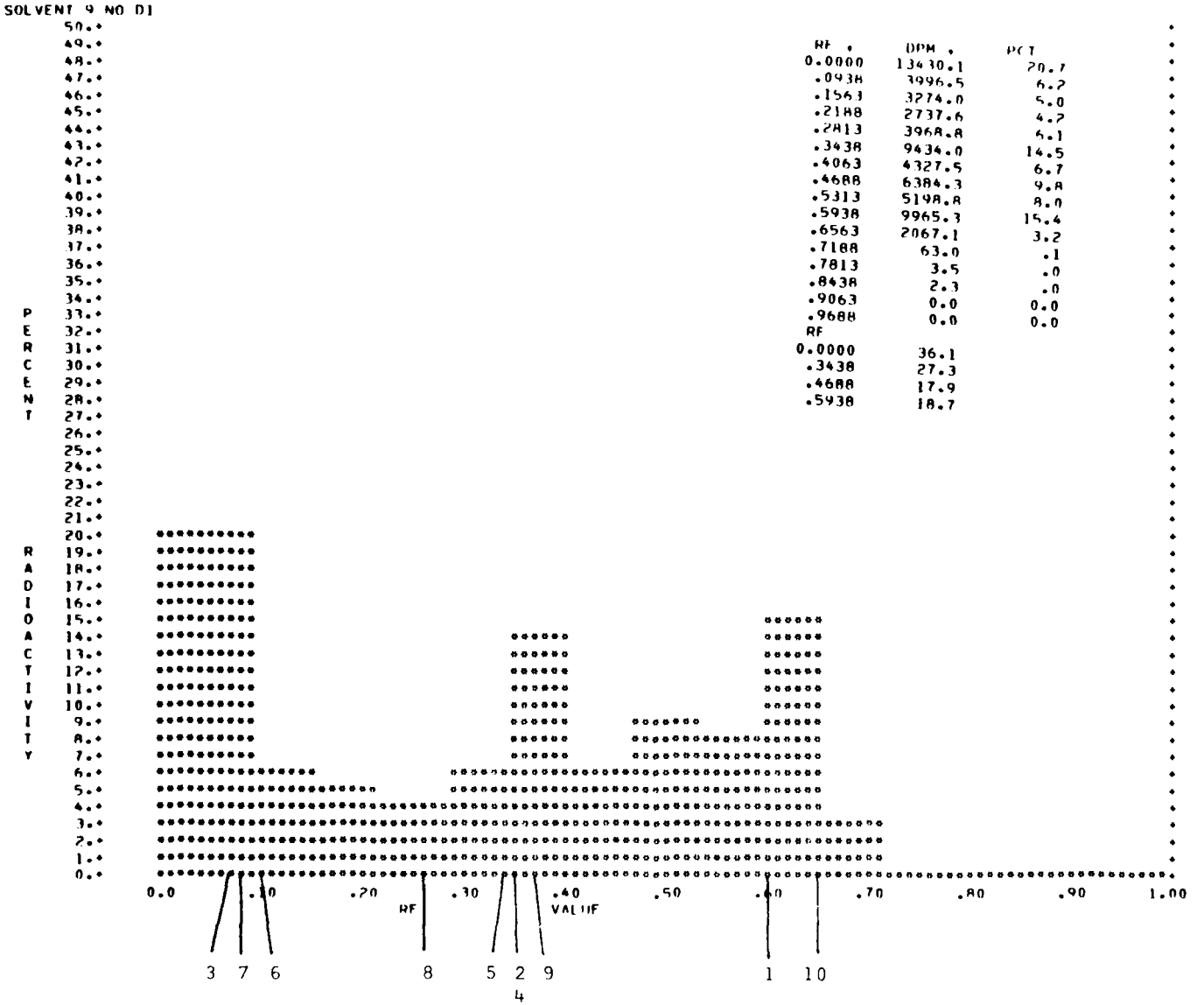

Figure 40-E_1: Solvent IX

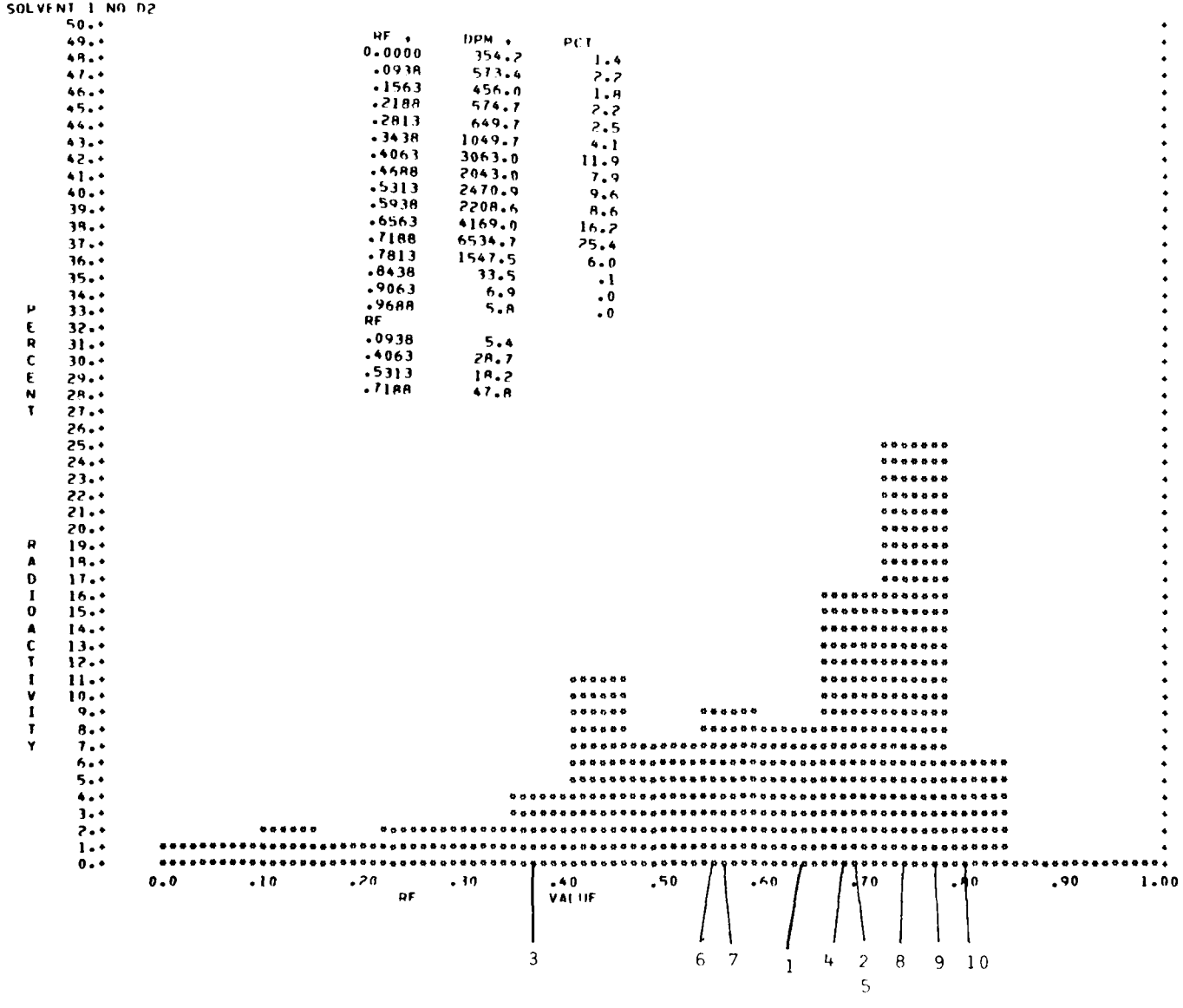

Figure 40-E_2: Solvent I

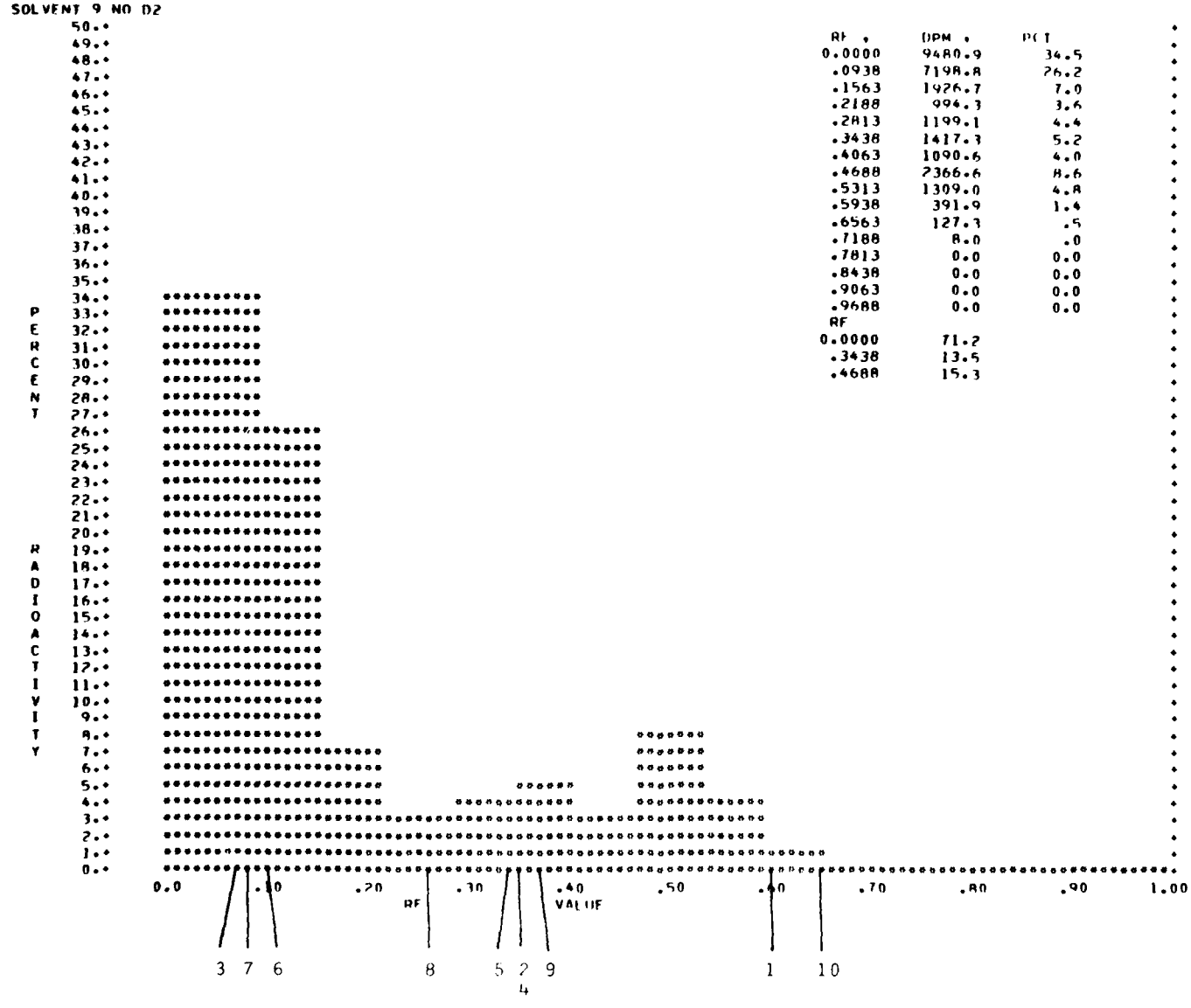

Figure 40-E_2: Solvent IX

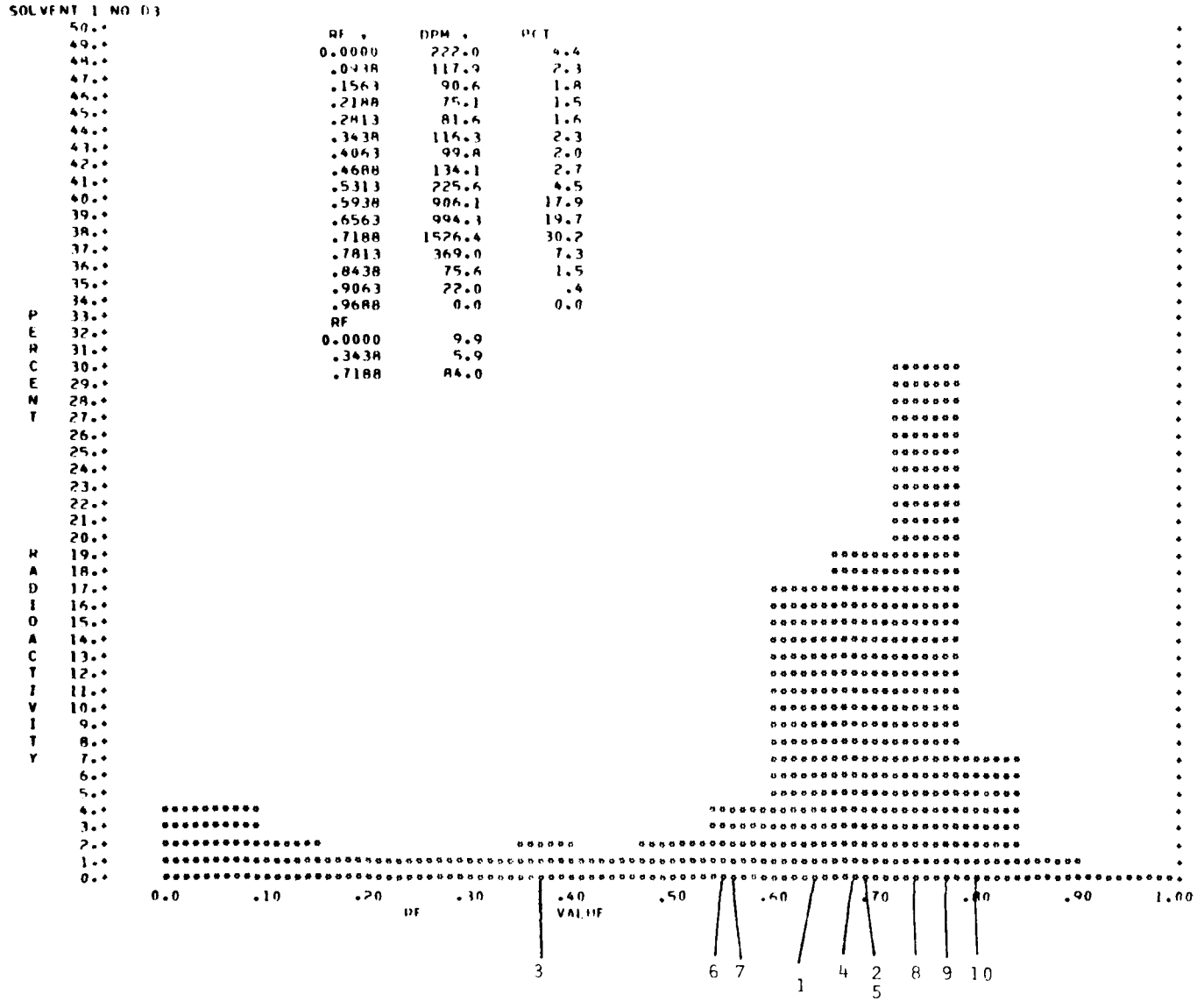

Figure 40-E3: Solvent I

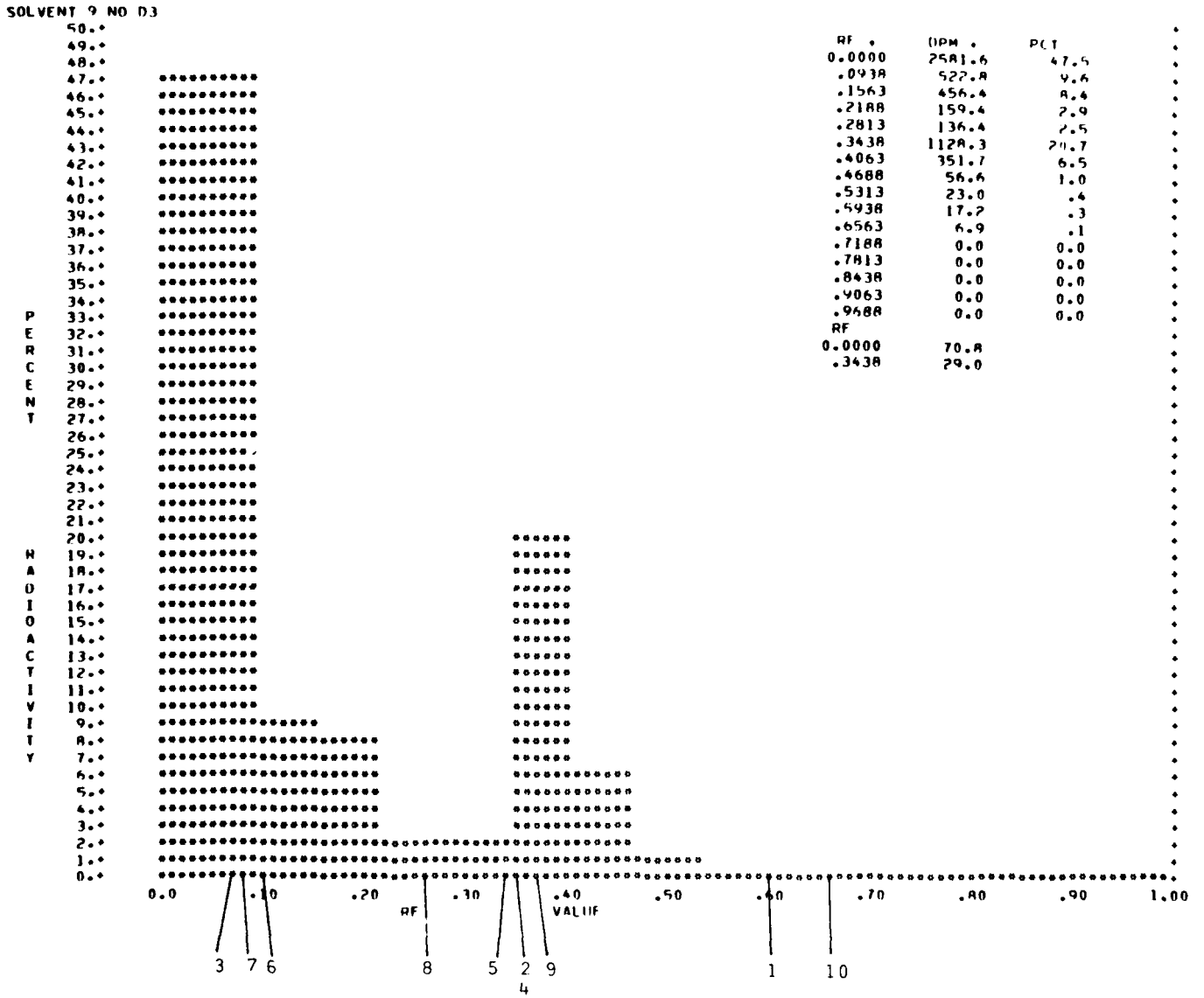

Figure 40-E_3: Solvent IX

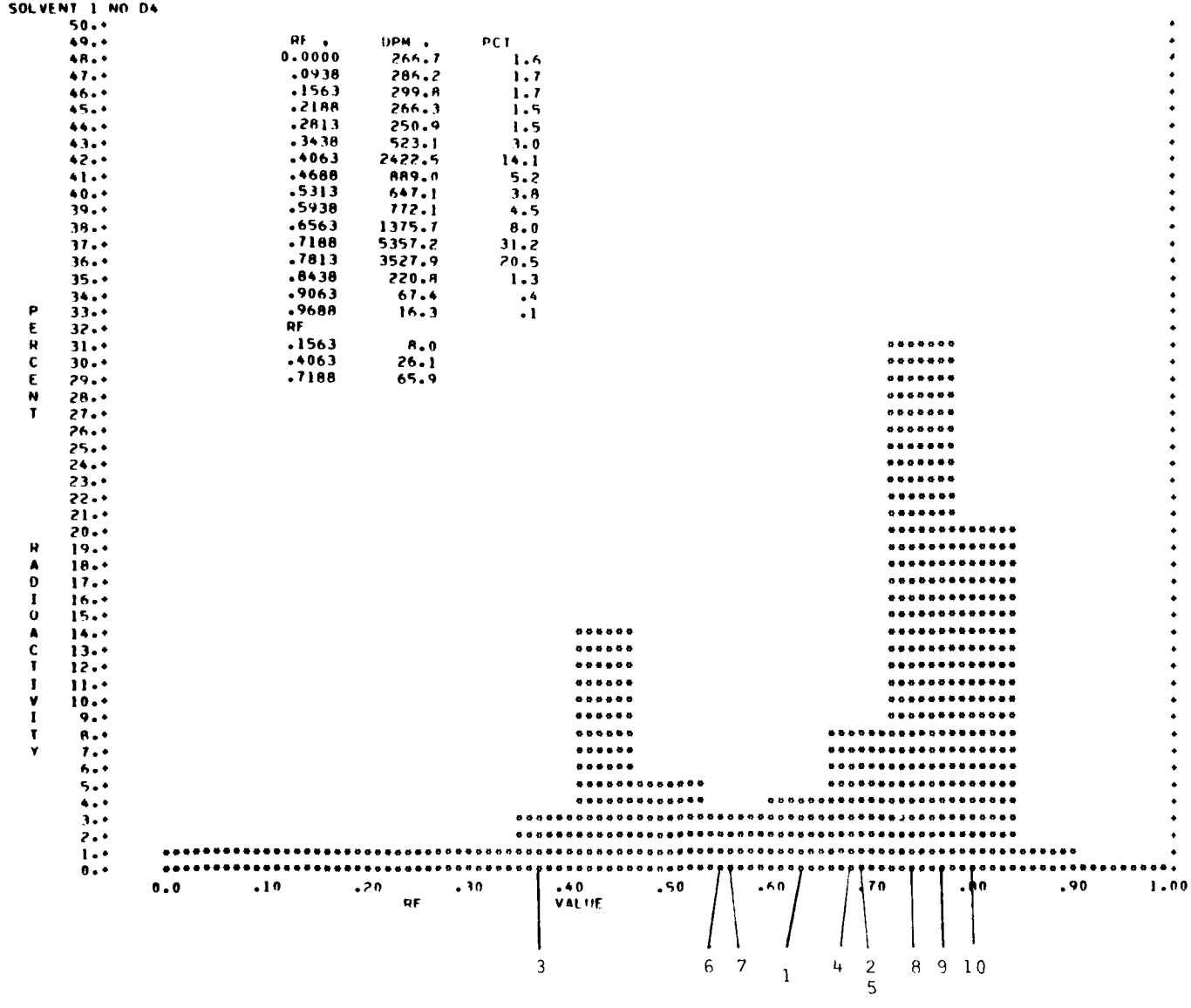

Figure 40-E_4: Solvent I

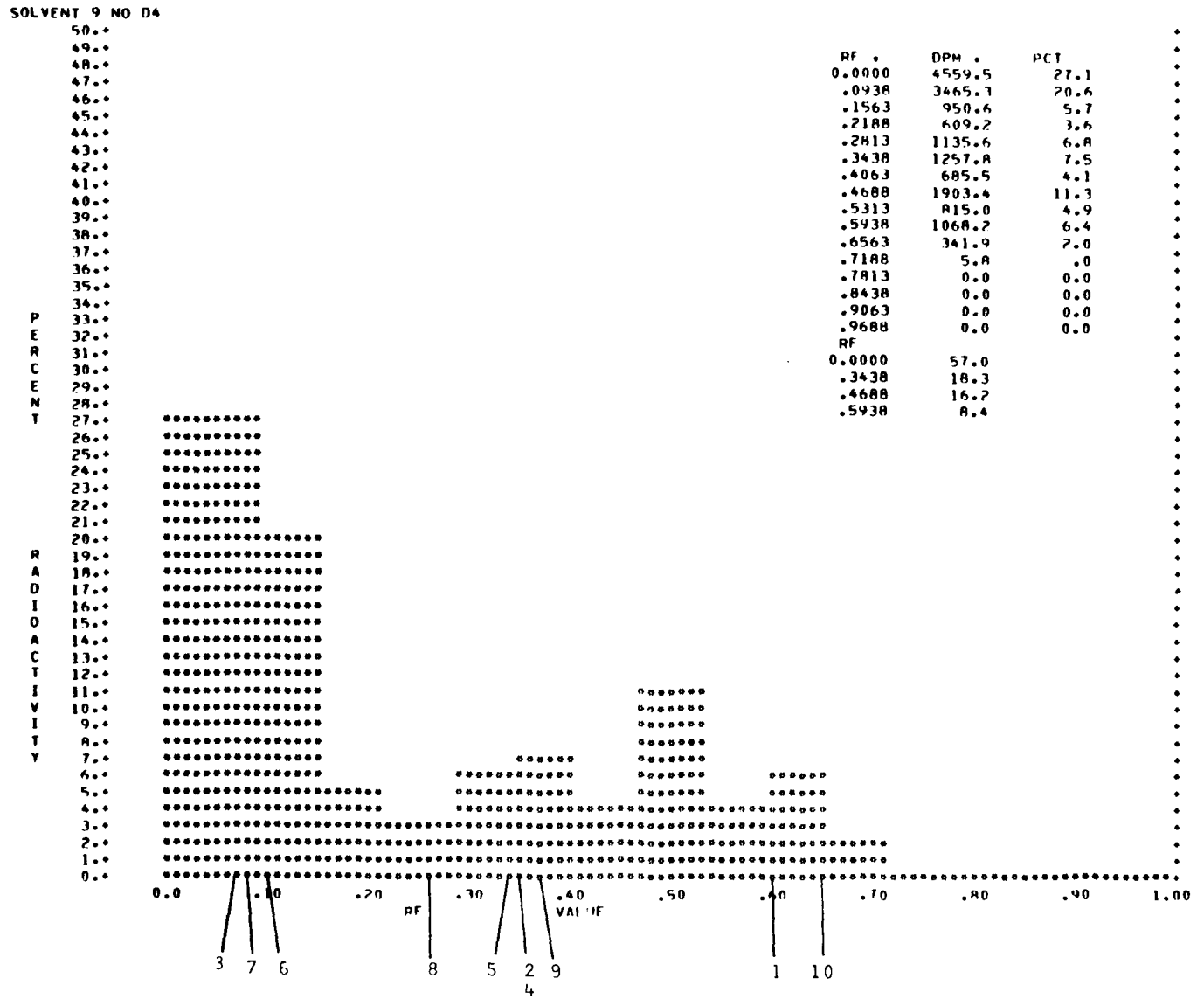

Figure 40-E4: Solvent IX

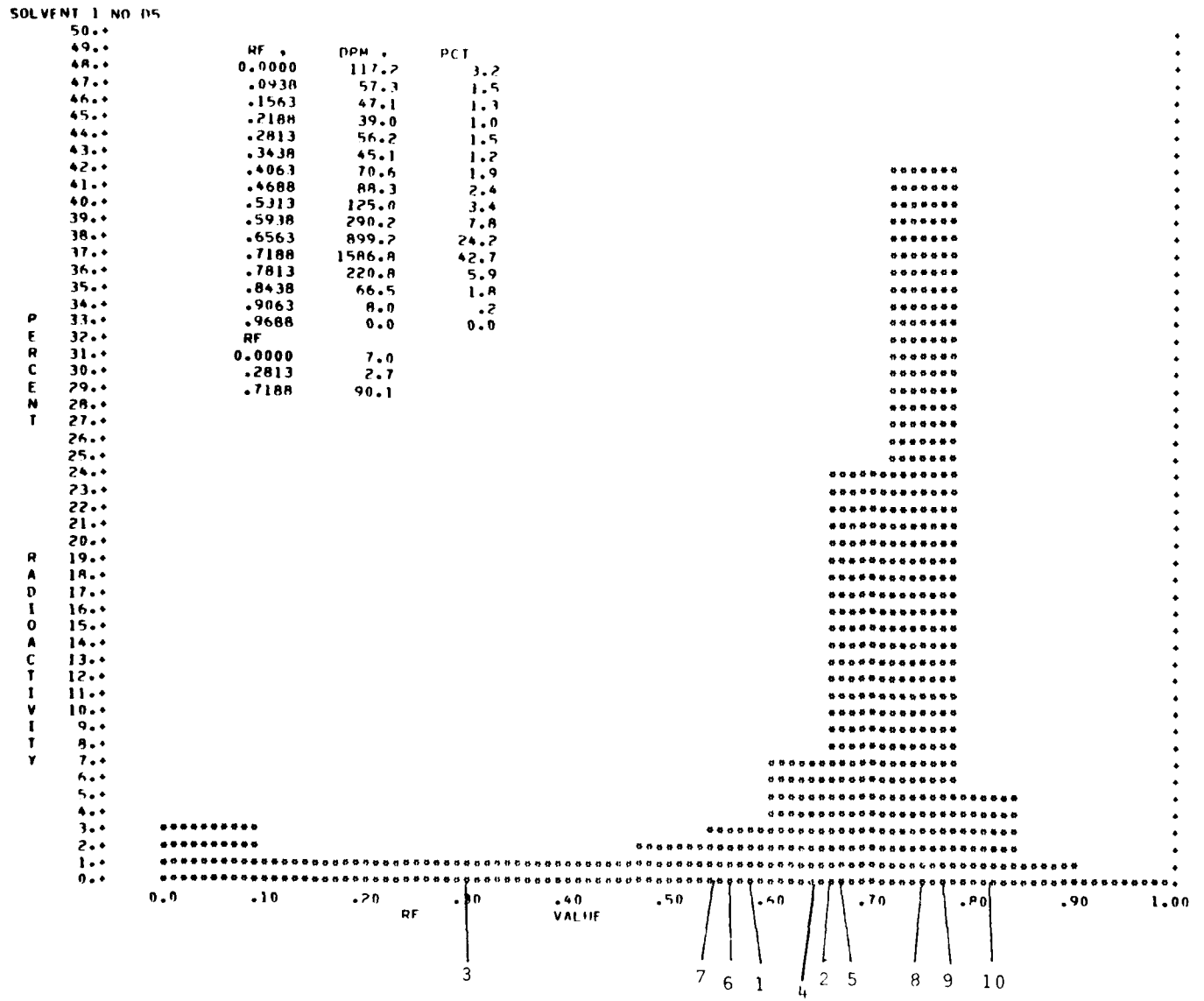

Figure 40-E5: Solvent I

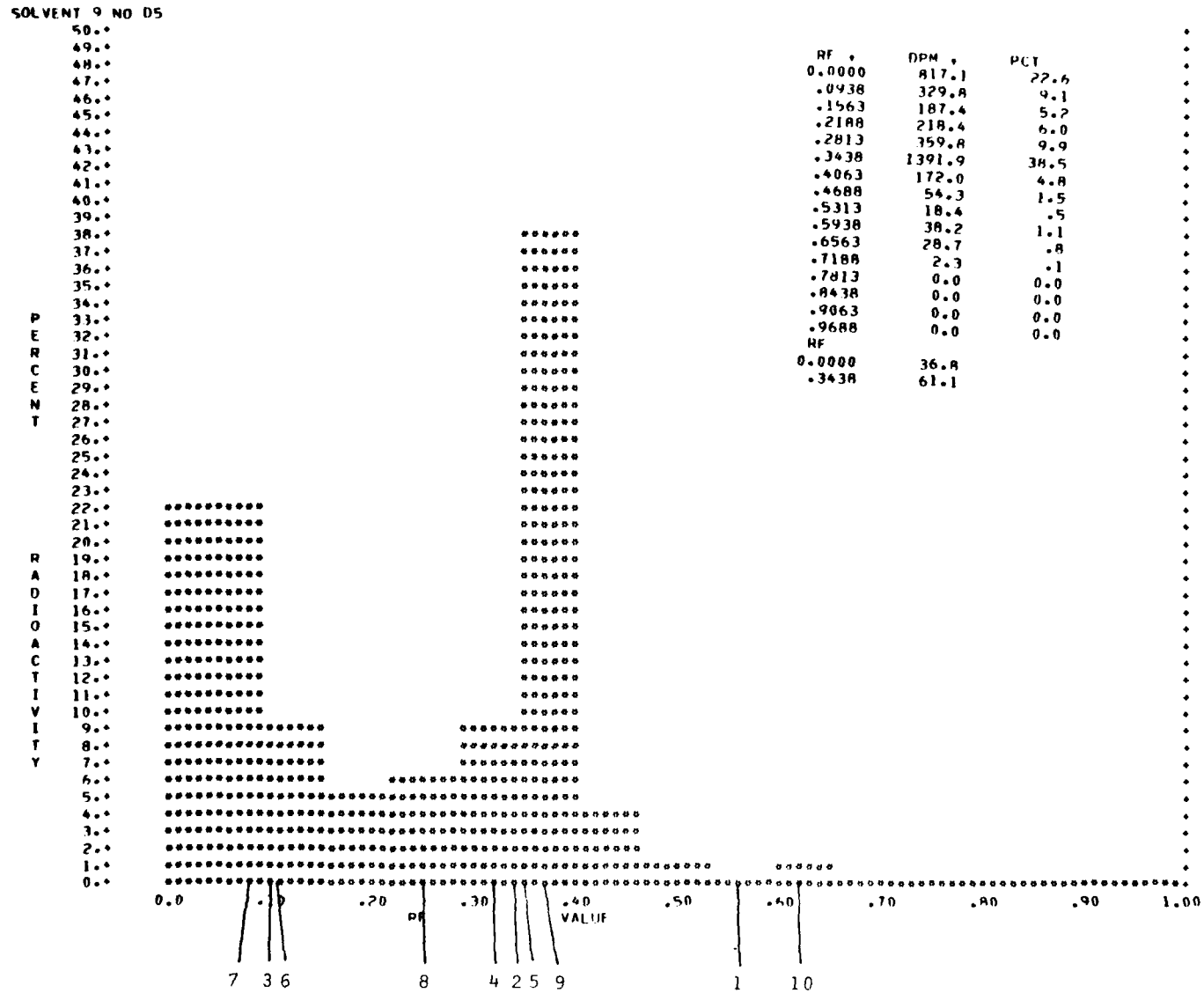

Figure 40-E_5: Solvent IX

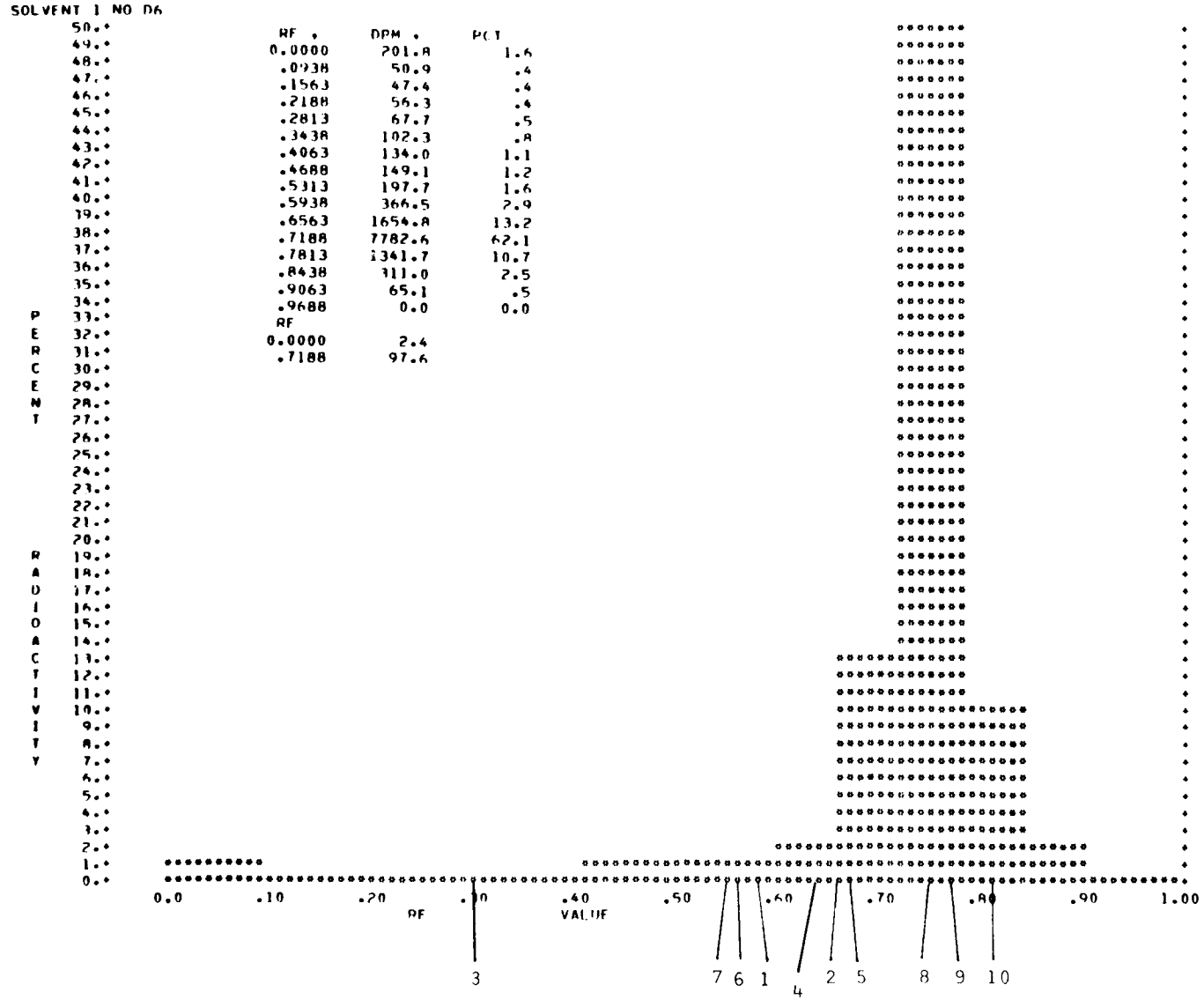

Figure 40-E_6: Solvent I

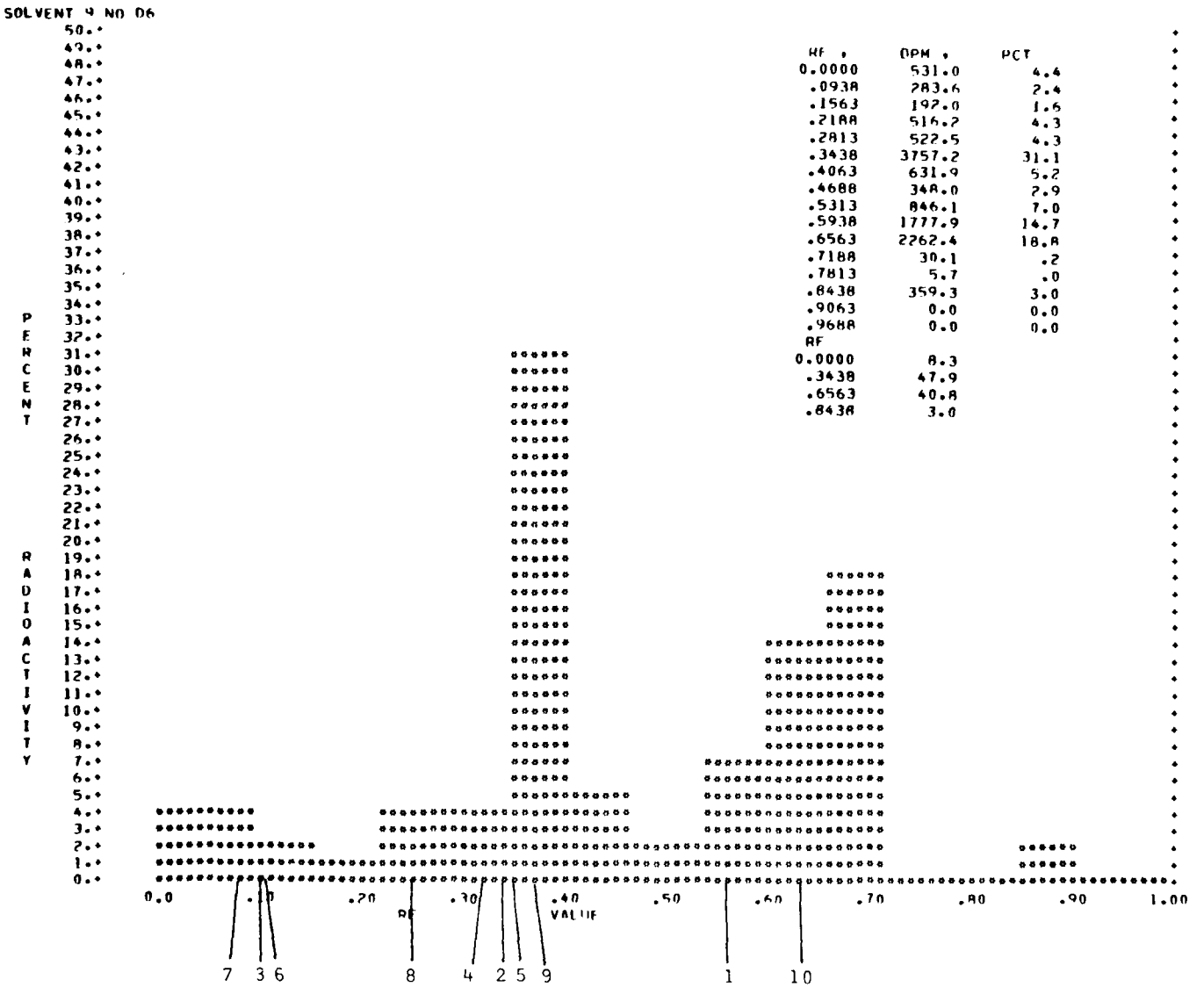

Figure 40-E_6: Solvent IX

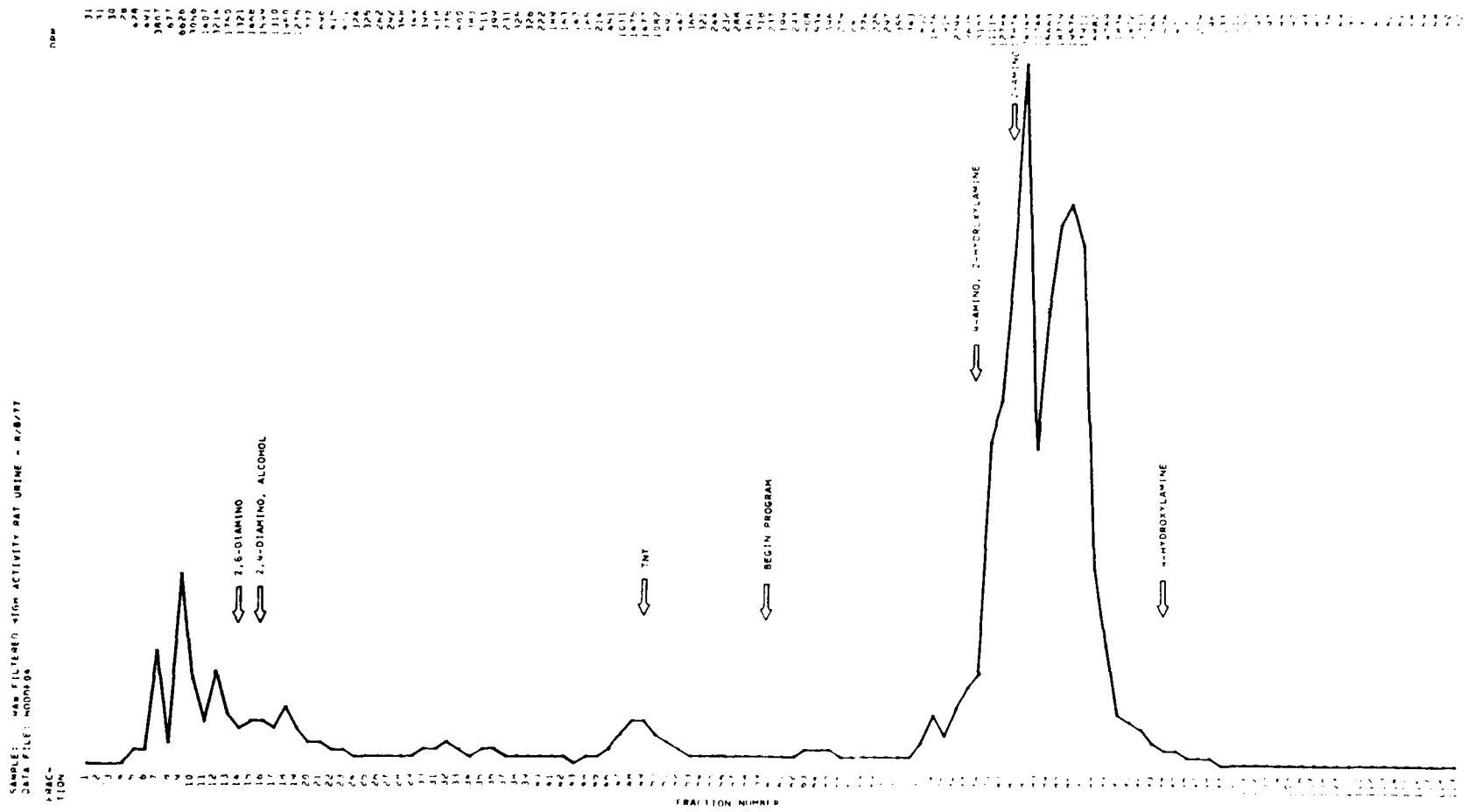

Figure 41 - HPLC of Rat Urine Obtained After Oral Administration of ^{14}C-TNT. Volume of urine injected was 100 µl and 0.8 ml fractions were collected.

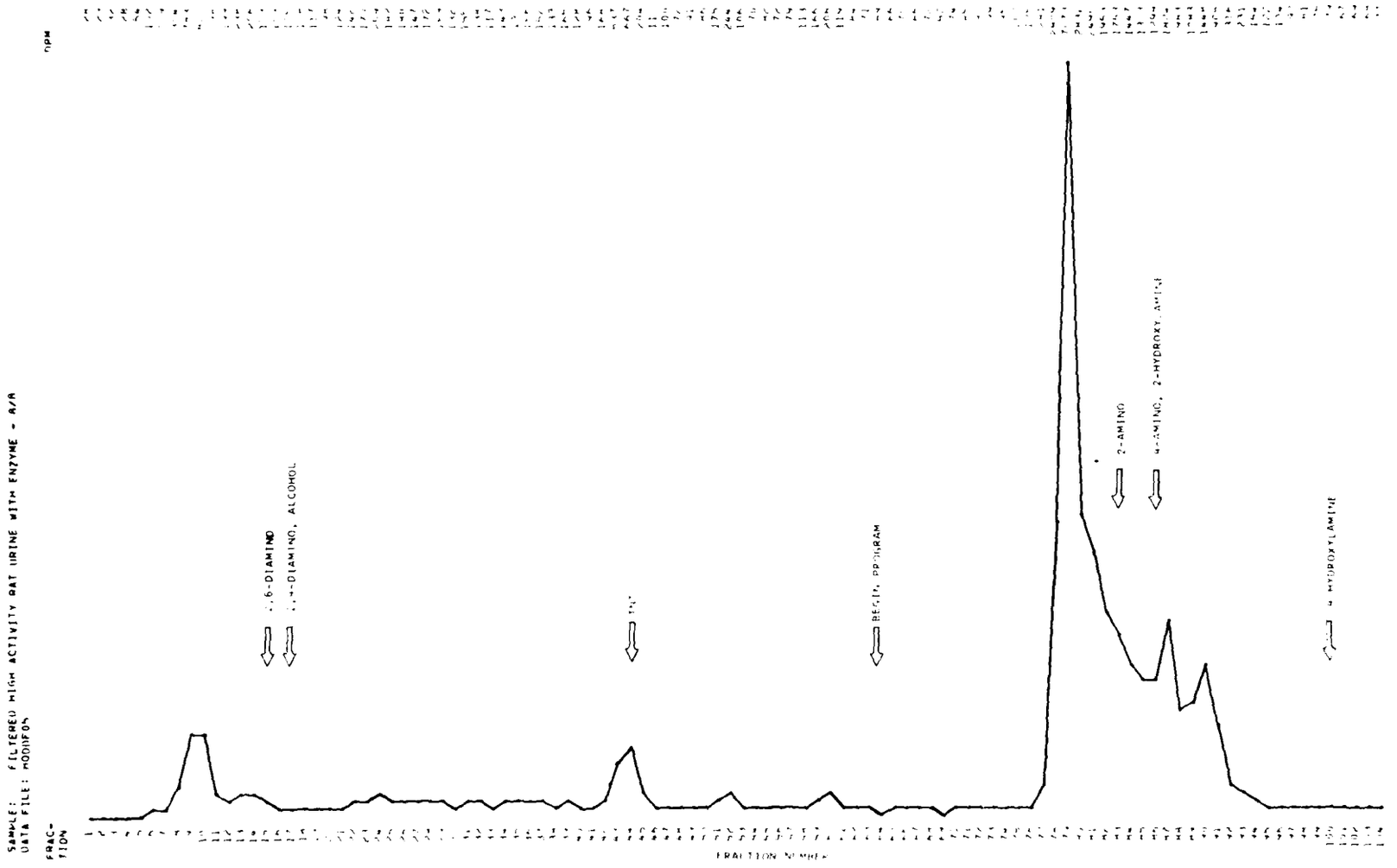

Figure 42 - HPLC of Rat Urine Obtained After Oral Administration of ^{14}C-TNT. Urine was treated with β-glucuronidase for 24 hr at 37° in the presence of acetate buffer (pH 5.0). Volume of urine injected was 25 µl and 0.8 ml fractions were collected.

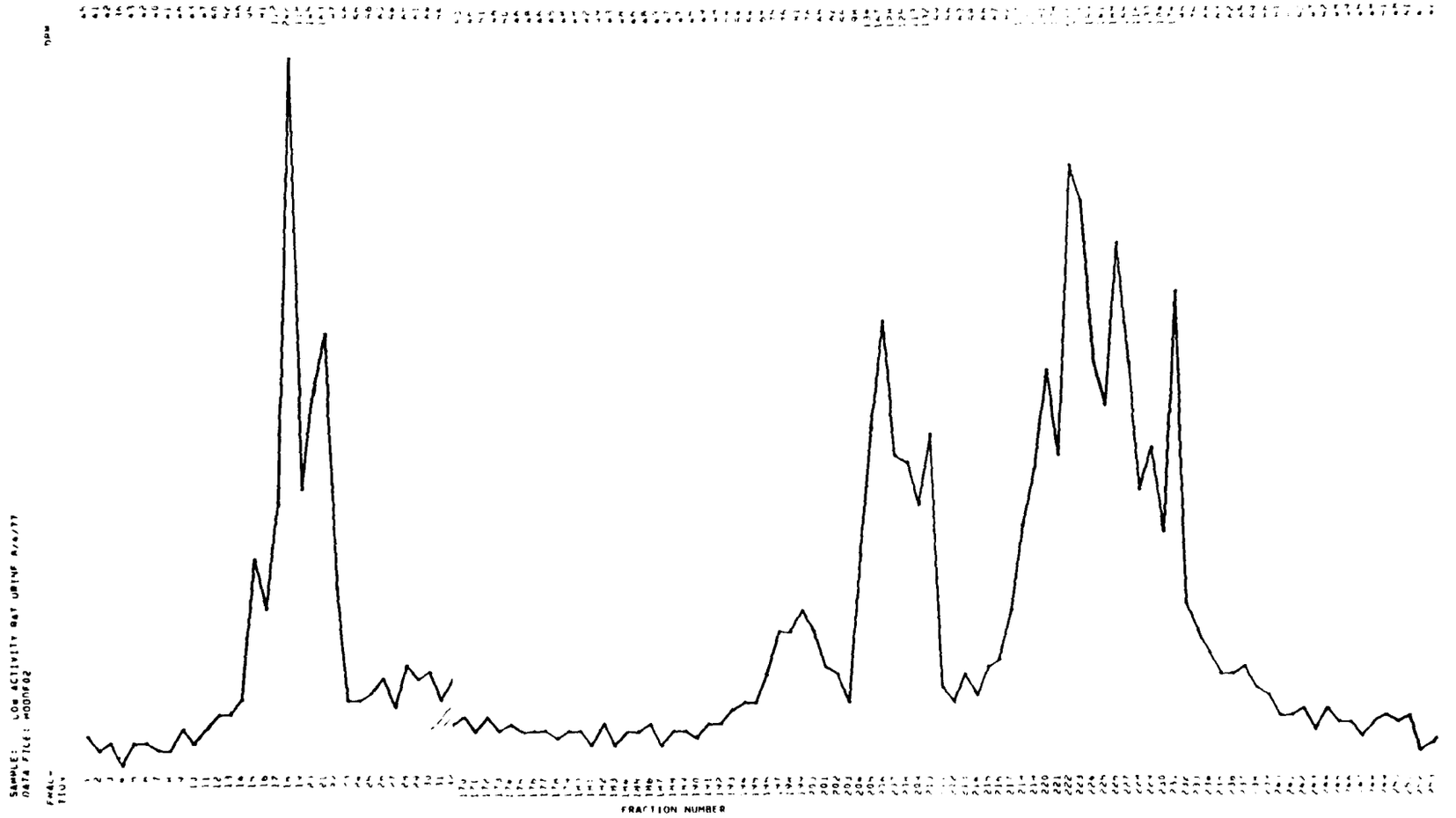

Figure 43 - HPLC of Rat Urine Obtained After Oral Administration of ^{14}C-TNT. Volume of urine injected was 20 µl and 0.4 ml fractions were collected.

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