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BIOCHEMICAL CHARACTERISTICS OF INSECT MICROSOMES

N- AND *O*-DEMETHYLATION*

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Abstract—The *N*-demethylation of *N,N*-dimethyl-*p*-nitrophenol carbamate and the *O*-demethylation of *p*-nitroanisole by the microsomes from housefly abdomens were studied.

Demethylation activity of male flies rises to a maximum at 4 days after emergence, while the maximum activity of females is not realized until 7–9 days after emergence.

Homogenization with a teflon pestle is more effective than a blender, and 0·20 M potassium phosphate buffer (pH 7·8) was found to be superior to all other media tested for the preparation of the microsomes. It was apparent that the ionic strength of the preparative medium is more important than either molarity or pH for obtaining microsomes of maximum *N*- and *O*-demethylating activity. During the subsequent incubation, 0·5 to 1·0% bovine serum albumin is required for maximum activity.

The pH optimum for both *O*- and *N*-demethylation is between 7·7 and 8·1 with added NADPH or with the generating system, and both reactions show the greatest activity at 30–33°. The apparent K_m for pNA is $9{\cdot}0 \pm 0{\cdot}4 \times 10^{-5}$ M and that for DpNC is $4{\cdot}0 \pm 1{\cdot}6 \times 10^{-4}$ M.

THE ENDOPLASMIC reticulum, which can be isolated *in vitro* as microsomes, is the center for the metabolism of many drugs and pesticides,[1,2] collectively referred to as "xenobiotics".[3] In order to proceed on such problems as insecticide metabolism, synergism, selective toxicity, and resistance, it is necessary to develop a more complete understanding of the microsomal detoxication systems of insects.[2,4,5]

Although mammalian microsomes have been the subject of intense study for several years, comparable preparations from insects have been achieved only recently. Early work was complicated by endogenous inhibitors[6–8] and reluctance to depart from the methods used for mammalian systems. However, within the last few years, investigators in many laboratories have been able to prepare insect microsomes which seem to reflect the *in vivo* system more accurately.[8–16] However, the methods used for studying microsomal metabolism in insects vary considerably from laboratory to laboratory, often with no apparent basis for the preference.

The present study is concerned with the effects of variations of both preparation and incubation procedures on *N*- and *O*-demethylation by housefly microsomes. These data, in addition to providing new information on *N*- and *O*-demethylation in the

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housefly, contribute toward a rational approach to the selection of methods for comparative toxicology.

MATERIALS AND METHODS

Chemicals. NADP, NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase (Type XI from Torula yeast) were obtained from Sigma Biochemicals. Bovine serum albumin, fraction V (BSA) was obtained from Armour Pharmaceuticals. All other chemicals were of reagent grade and were used without further purification.

Substrates and products. *N,N*-dimethyl-*p*-nitrophenol carbamate (DpNC) was a gift from the Union Carbide Chemical Company and was recrystallized from ethanol immediately prior to use. DpNC was added to the incubation flasks in ethanol solution and the ethanol evaporated.

Both *p*-nitroanisole (pNA) and *p*-nitrophenol (pNP) were obtained from Eastman Organic Chemicals and were recrystallized before use. A solution of 2·0 μmoles/ml of pNA in 0·5% ethanol was made from an ethanol solution containing 400 μmoles/ml. Such a solution was stable at room temperature for several weeks.

Aniline obtained from Eastman Organic Chemicals was redistilled, retaining the fraction with an n^d_{20} of 1·585. An aqueous solution of 100 μmoles/ml was used for assays. *p*-Aminophenol, obtained from Eastman Organic Chemicals, was recrystallized and standard solutions prepared immediately prior to use.

Enzyme source. Except in age and sex studies, all microsomes were prepared from the abdomens of 6–8-day-old female houseflies of the standard Chemical Specialties Manufacturer's Association (CSMA) strain originally obtained from Union Carbide Chemical Company and maintained at this University since 1962. Adults were fed on milk and sugar cubes and reared in a 16:8 (light:dark) photoperiod. All tissue fractionation steps were carried out on ice with prechilled equipment and solutions. Abdomens from 100 female flies were homogenized in 10 ml of potassium phosphate buffer (0·2 M, pH 7·8) by 10 strokes in a cone-driven teflon pestle in a fitted Pyrex tube, except when the method of homogenization was studied. In this case, 200 abdomens in 20 ml potassium phosphate buffer were homogenized in a Servall Omni-Mixer for 45 sec with the variable resistor set at 90. In some experiments homogenizing media were varied for comparison.

The homogenate was filtered through cheesecloth and centrifuged for 15 min at 12,100 *g* in a Servall refrigerated centrifuge. The supernatant was filtered through glass wool to remove fat and gross debris and centrifuged for 1 hr at 105,000 *g* in a Spinco model L-2 ultracentrifuge.

In all studies, the supernatant was decanted and the microsomes resuspended to the equivalent of 30 flies/ml in tris-HCl buffer (0·1 M pH 7·9) containing 2% BSA. Microsomes were used immediately after preparation, except in the storage experiments. Throughout this investigation, in comparisons of preparative methods, microsomal pellets were treated identically from the final resuspension through the assay procedure.

Assay of enzyme activity. The reaction was initiated by the addition of enzyme (1·0 ml) to 3·0 ml of the reaction mixture at 30°. The reaction mixture was incubated in open 25-ml Erlenmeyer flasks in a Dubnoff Metabolic shaker for 30 min at 30°. For temperature studies, Eberbach Incubator-Shakers were also used, and each was adjusted to the same agitation speed.

The incubation mixture contained microsomal suspension equivalent to 30 flies (approximately 2 mg microsomal protein as determined by the method of Lowry *et al.*[17]) and either 2·0 μmoles of pNA, 5·0 μmoles of DpNC, or 100 μmoles of aniline in a total volume of 4·0 ml. In addition, the following cofactors were present in the indicated concentrations: NADP, 5×10^{-4} M; glucose-6-phosphate, $2{\cdot}5 \times 10^{-3}$ M; $MgCl_2$, $7{\cdot}5 \times 10^{-3}$ M; tris buffer (pH 7·9), 5×10^{-2} M (final concentration); BSA, 5 mg/ml; glucose-6-phosphate dehydrogenase, 0·25 units/ml.

For determination of *p*-nitrophenol, the product of *O*-demethylation of pNA as well as the hydrolysis product of the *N*-demethylation metabolite of DpNC,[18] the reaction was stopped with 1 ml of N HCl. 1 N HCl rapidly degrades the *N*-demethylation metabolite of DpNC to *p*-nitrophenol while the substrate is stable under these conditions.[18] The acidified contents were shaken with an equal volume (5·0 ml) of chloroform and centrifuged. Three ml of the chloroform layer were then shaken with 3 ml of 0·5 N NaOH and centrifuged. The optical density at 400 mμ, of the NaOH layer (containing *p*-nitrophenate ion), was determined in a Beckman DB spectrophotometer.

Aniline hydroxylation was determined by measuring the production of *p*-aminophenol after the method of Imai and Omura (in Schenkman *et al.*[19]).

RESULTS AND DISCUSSION

General considerations. Due primarily to the presence of endogenous inhibitors in the head and thorax, [6–8] many workers have found that microsomes prepared from housefly abdomens are preferable to those prepared from other body regions or from whole flies. For this reason, abdomen microsome preparations were used exclusively in this work.

Apart from some type of post-emergence increase, there appears to be no consistent trend reported in the literature concerning the effects of age and sex on microsomal enzyme activity.[8,11,20] Therefore, these effects must be considered in light of the particular methods being employed and the reaction being assayed. As can be seen from Fig. 1, both *N*- and *O*-demethylation are quite dependent on the age and sex of the fly when expressed on a "per fly" basis. The earlier peak activity of the male flies as compared to the female flies may be due to earlier maturation[21] and subsequent earlier senescence; this is supported by the observation that, in flies reared under standard rearing conditions, the male population is depleted before the female population, and after 2 days, many of the males are flightless due to lost and damaged appendages.

It has also been found that certain strains of resistant flies have considerably higher microsomal oxidase activity than do susceptible flies.[8,22–26] A diazinon-resistant strain of flies, obtained from Dr. A. S. Perry, has been shown to have a 4- to 5-fold higher level of both *N*- and *O*-demethylase activity.[26] However, the studies reported in this communication utilized the susceptible CSMA strain. The oxidase acitivity of this strain toward the substrates used was adequate after initial experiments delineated some of the conditions necessary.

Methods of preparation. In agreement with Schonbrod and Terriere,[12] the method of homogenization played an important role in the oxidase activity of the resulting microsomes as shown in Table 1. In each case, the activity was higher in microsomes

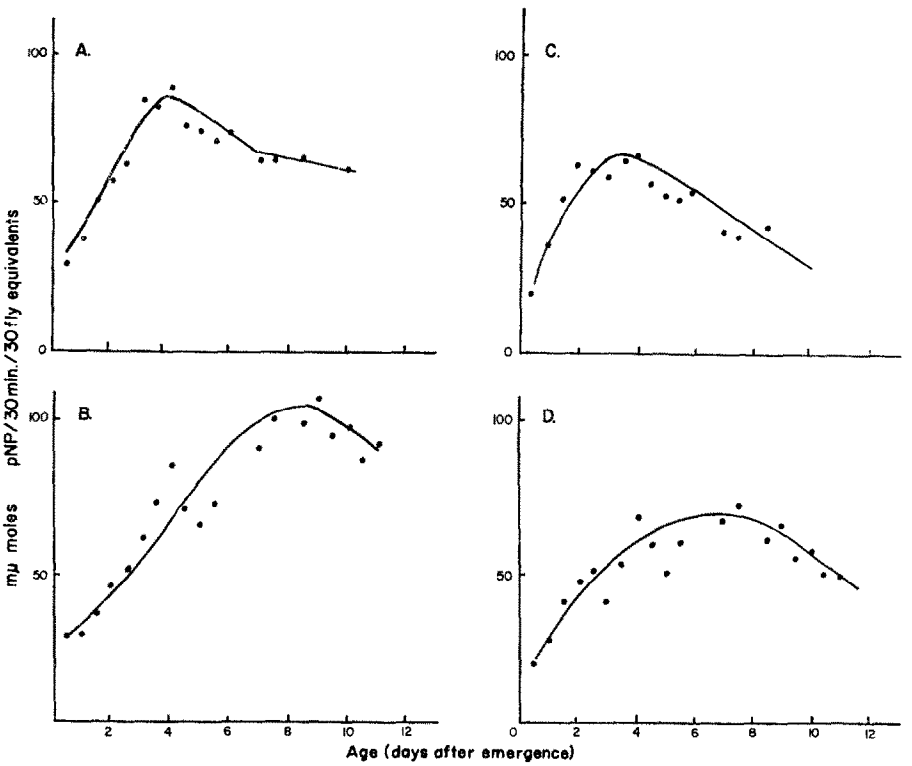

FIG. 1. Variation with age of demethylation in microsomes from male and female fly abdomens. Results in mean m-μmoles pNP produced from four determinations on two groups of flies: (A) Male, *O*-demethylation; (B) Female, *O*-demethylation; (C) Male, *N*-demethylation; (D) Female *N*-demethylation.

TABLE 1. EFFECT OF METHOD OF HOMOGENIZATION OF DEMETHYLATIONS AND ANILINE HYDROXYLATION BY RESULTING MICROSOMES

	m-μmoles product/30 fly equivalents/30 min					
	Homogenized with Teflon pestle			Homogenized in Omni-mixer		
Preparation no.	pNA	DpNC	Aniline	pNA	DpNC	Aniline
1	111	87	50	96	81	45
2	109	77	40	75	70	35
3	99	86	42	78	65	27
4	112	—	—	59	—	—
5	113	—	—	91	—	—
Mean	108·8	83·3	44·0	79·8	72·0	35·7

* Average of two determinations for each preparation.

from homogenates prepared using a teflon pestle than those prepared in the Omni-Mixer; in addition, the use of the teflon pestle greatly reduced the variation from one microsomal preparation to the next.

Differential centrifugation was carried out using the methods of Cassidy *et al.*,[27] and enzyme assay indicated an intracellular distribution of demethylase activity as shown in Table 2. The activity present in all other particulate preparations is probably

TABLE 2. INTRACELLULAR DISTRIBUTION OF DEMETHYLATION ACTIVITY

	DpNC		pNA	
Fraction	m-μmoles	*%	m-μmoles	%
Whole homogenate	53	—	56	—
Nuclei and debris (R-2000)†	16	15	18	16
Mitochondria (R-12,100)	18	17	17	15
Mitochondrial Supernatant (S-12100)	80	—	79	—
Microsomes (R-100,000)	72	67	76	68
Soluble (S-100,000)	2	1	0	0

* Per cent of total for debris, mitochondrial, microsomal and soluble fractions.

† R = residue and S = supernatant at the particular g force shown.

due to contamination with microsomal fragments. It is clear, however, that the activity is primarily located in the microsomal fraction. The low activity in the whole homogenate is probably due to endogenous inhibitors which can be removed by centrifugation.[7]

Five different media for the initial homogenization were tested and 0·25 M potassium phosphate buffer (pH 7·8) was found to be superior to 0·25 M sucrose, 0·25 M sucrose plus 0·15 M phosphate, 1·15% potassium chloride, and 0·71 M potassium chloride. Figure 2 shows that 0·2 M phosphate buffer (ionic strength, 0·57) is the optimum concentration of this buffer for the preparation of microsomes. In a separate experiment, it was shown that KCl buffered with tris is equivalent to 0·2 M potassium phosphate buffer if the pH and ionic strengths are the same. The close agreement of the peaks for both substrates and both media shown in Fig. 2 clearly establishes the importance of the ionic strength of the medium of preparation, since the molar concentrations of phosphate and chloride were quite different. It can also be seen that the microsomal demethylases are more sensitive to fluctuations in the ionic strength of potassium phosphate during preparation than to similar changes in the ionic strength of KCl.

Microsomes resuspended in 2% BSA in tris, as described in Materials and Methods and stored at 0–4°, lose considerable activity within 2 days. Although the loss of activity is approximately parallel from 8 to 48 hr, the rate of activity loss during the first 8 hr, and especially during the first 2 hr, is dependent on the molarity of the buffers in which the abdomens were homogenized. The more rapid loss of activity of

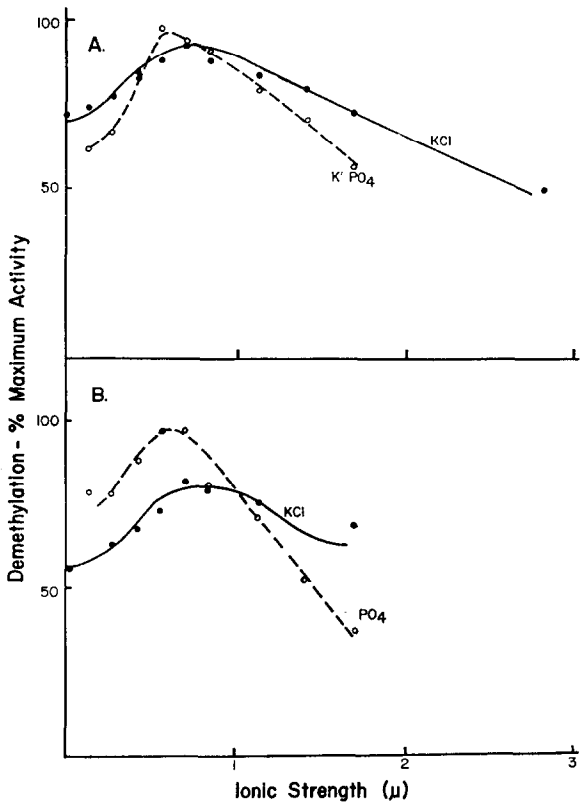

FIG. 2. Effect of potassium phosphate (pH 7·8) and KCl (pH 6·9) ionic strength in preparative medium on (A) *O*-demethylation and (B) *N*-demethylation by resulting microsomes. Maximum activity is at 0·20 M $KHPO_4$ ($\mu = 0{\cdot}57$).

the microsomes prepared in the higher ionic strength buffers could readily distort the effects observed in Fig. 2 if the microsomes were stored before use. These differences, although small, are consistent and reproducible.

The pH of the medium in which the microsomes are prepared does not appear to be a major factor between pH 7 and 8 if the buffers used are adjusted to the same ionic strength.

It has been reported[8,28] that 1·5% BSA is required in the incubation mixture to obtain maximum activity from housefly microsomes but that BSA is not required during preparation. It was shown that when microsomes are prepared in 0·20 M phosphate buffer containing BSA they are less sensitive to BSA addition during incubation; however, maximum activity can be achieved, with or without BSA in the preparative step, by including 0·5–1·0% BSA in the incubation medium. The lower activity of microsomes incubated in 2·5% BSA is probably due to a lowering of the pH or reduced availability of the lipophilic substrates to the microsomes.

Conditions during incubation. A study of enzyme concentration showed that under the conditions described, the enzyme was rate limiting up to an equivalent of 50 flies per flask. Subsequent incubations employed an equivalent of 30 flies per reaction, and

the normal range of product for either substrate was 100–120 m-μmoles pNP produced per 30 min.

Although the higher ionic strength necessary for preparation of the microsomes is more conveniently obtained with phosphate buffer, preliminary experiments showed that 0·05 M tris was superior to 0·1 M phosphate for the incubation medium. Recent experiments show that if the pH and ionic strength are carefully controlled, either buffer serves equally well for both preparation and incubation of the microsomes.

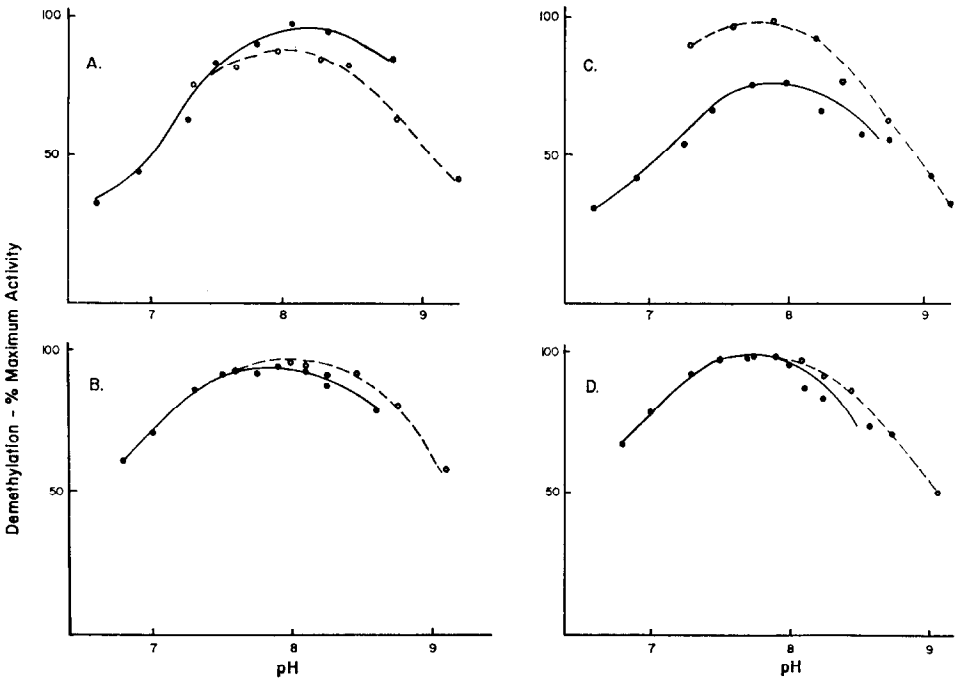

FIG. 3. Effect of pH of incubation medium on microsomal demethylations with and without the glucose-6-phosphate–NADPH generating system. (A and B) *O*-demethylation; (C and D) *N*-demethylation. (A and C) generating system; (B and D) NADPH. Solid line is 0·05 M tris; Broken line is 0·05 M 2-amino-2-methyl-1,3-propanediol.

Figure 3 shows that the pH optima for both reactions are between 7·7 and 8·1 either with an NADPH-generating system or with added NADPH alone. Thus, any effect that pH may exert on the generating system is not reflected in the demethylation reactions. In all four cases, the pH remained essentially the same throughout the reaction. These values agree with the optimum of 7·8–8·2 for aldrin expoxidation reported by Brooks and Harrison.[29]

The effect of temperature on microsomal *O*- and *N*-demethylation can be seen in Fig. 4. Both reactions exhibit the greatest activity at 30–33°. The importance of determining the optimum temperature when studying xenobiotic oxidation has previously been emphasized by Terriere.[30]

The effect of added NADPH and NADP plus an NADPH-generating system is shown in Fig. 5. The lower concentrations (0–2 $\times$ 10^{-4} M) of NADPH or NADP plus the generating system result in a dramatic increase in the reaction rate which then

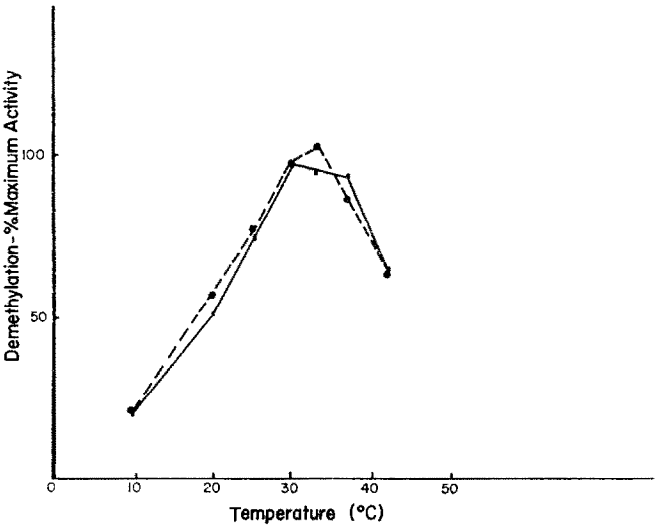

FIG. 4. Effect of temperature on microsomal demethylations during a 30-min incubation. Solid line is DpNC; broken line is pNA.

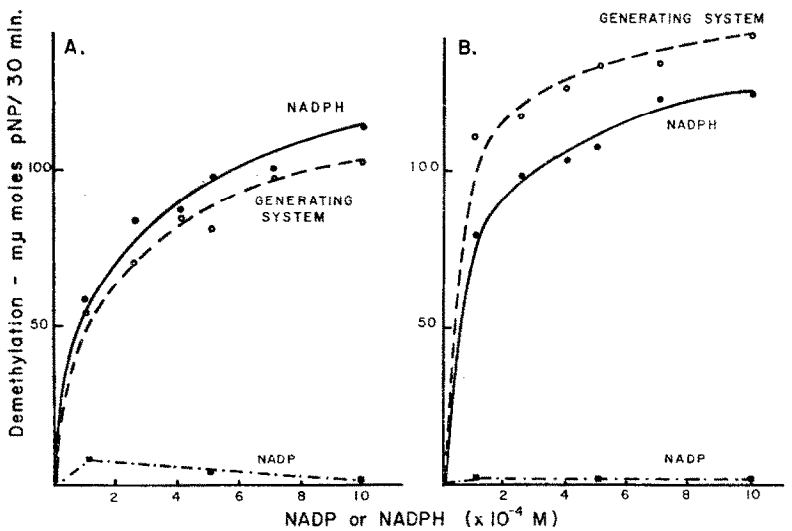

FIG. 5. Effect of cofactor concentration on microsomal (A) *N*-demethylation and (B) *O*-demethylation.

levels off rapidly. The difference between the generating system and added NADPH was generally small.

NADH alone cannot satisfy the requirements as can be seen from the lower line on each graph; in fact, NADP alone appears to inhibit the low level of activity due to endogenous reducing power. This may be similar to the known inhibition of drug metabolism in mammals by NADP.[1]

Glucose-6-phosphate was added in excess (10 μmoles) as shown by the fact that no significant change in *N*-demethylase activity occurred between 2 and 10 μmoles.

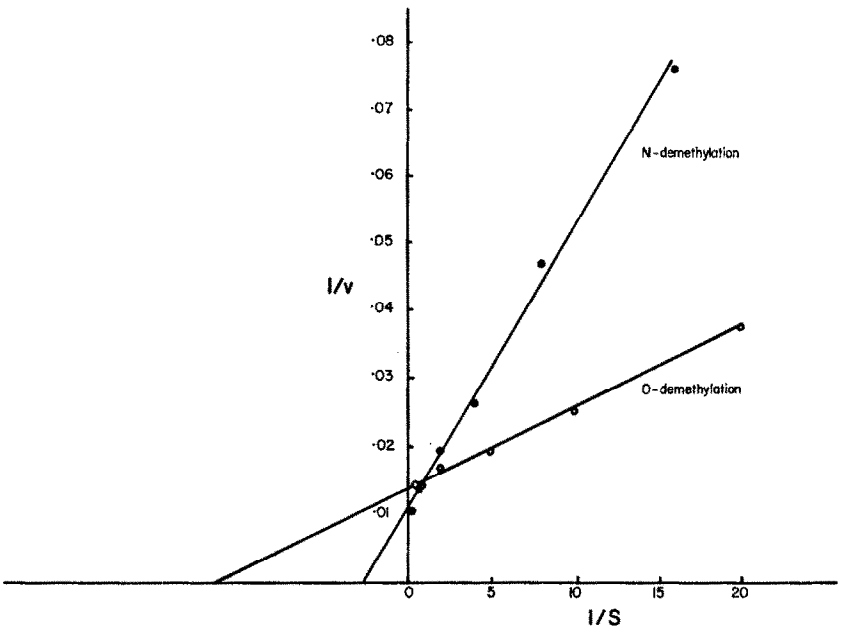

FIG. 6. Lineweaver–Burk plots of microsomal demethylation activity v, substrate concentration, S, substrate added (μmoles); v, pNP produced (m-μmoles). *N*-demethylation, 15 min incubation period; *O*-demethylation, 10 min incubation period.

Activity was increased slightly by increasing the amount of glucose-6-phosphate dehydrogenase added up to 4-fold. Eighty per cent of the maximum activity for either demethylase could be retained without exogenous magnesium chloride, and magnesium sulfate could replace magnesium chloride at all concentrations. Concentrations of magnesium ion greater than 1×10^{-2} M resulted in reduced activity.

Figure 6 shows the reciprocal plots for representative K_m determinations for pNA and DpNC incubated with female housefly microsomes. Lines were fitted by the least-squares method, and the values given are the means of three completely independent determinations. The K_m for housefly microsomal DpNC *N*-demethylase was $4{\cdot}0 \pm 1{\cdot}6 \times 10^{-4}$ M and the V_{max} was 348 ± 80 m-μmoles pNP produced/30 fly equivalents/hr. The K_m for pNA *O*-demethylase was $9{\cdot}0 \pm 0{\cdot}4 \times 10^{-5}$ M and the V_{max} was 516 ± 126 m-μmoles pNP/30 fly equivalents/hr.

Given the same enzyme preparation, pNA yielded a greater amount of product than did DpNC, even though present in a lower concentration, due to a greater affinity for the microsomal system. Variations in reaction rate from one group of flies to the next were common so that, when day-to-day comparisons were to be made, the results often had to be expressed as a percentage of the maximum activity for each enzyme preparation.

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