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A Simple Method for Screening Petroleum Effluents by *In Vitro* Enzyme Inhibition[1]

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With the need to evaluate the environmental hazards of industrial wastes, there is increased demand for methods for quickly screening industrial effluents to determine if more sophisticated tests are needed, and, if they are, to establish priorities. While the animal systems currently being used for biological assessment are the best criteria for the final judgment of toxicant effect, simpler and less expensive methods for an initial on-site screening may be useful. In this regard, the inhibition of in vitro enzyme activity by industrial effluents has recently received some attention (CRIPPS and REISH 1973, JACKIM 1974). Enzyme inhibition, in fact, may be an underlying cause of the toxicity to animals (CRIPPS and REISH 1973; HEITZ et al. 1974). Although the mechanism for the toxicity of pollutants remains obscure, the available evidence points toward a metabolic inhibition. This is particularly true of chronic toxicity in which sublethal levels of toxicant express their effect over an extended period of time (JACKIM 1973, 1974; PAYNE 1976).

The present research was aimed at developing a rapid and inexpensive method for screening of petroleum effluents. A procedure is described for determining the extent of enzyme inhibition by a simulated petroleum effluent which contained "conventional contaminants" at maximum levels allowed to exist in a refinery effluent meeting 1977 guidelines (USEPA 1973).

MATERIALS AND METHODS

Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) type XV, isolated from Baker's yeast was purchased from Sigma Chemical Co., St. Louis, Missouri. The simulated petroleum effluent, otherwise known as the arbitrary reference mixture (ARM; BUIKEMA et al. 1976), contained 10 mg NH_4Cl, 0.25 mg K_2CrO_4, 10 mg No. 2 fuel oil, 0.1 mg phenol, 0.17 mg $Na_2S \cdot 9H_2O$ and 20 mg kaolinite in one liter diluent. The mixture containing the above concentrations was referred to as 1X ARM. At higher concentrations of ARM each component was increased by the same proportion (i.e., at 2X ARM all

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concentrations were doubled). Soft water diluent was carbon dechlorinated, aerated, Blacksburg tap water. The ranges of chemical parameters for Blacksburg tap water were: 35 to 45 mg/l hardness, 40 to 50 mg/l alkalinity, pH 7.0 to 7.4, and the ratio of magnesium to calcium was 3:10. The seawater diluent was 25% synthetic seawater (9 ppt) made from Dayno Synthetic Sea Salts (Dayno Sales Co.).

Inhibition of enzyme activity was tested by adding 10 µl (12 µg proteins or 2.4 enzyme units) of G6PDH and 1 ml of ARM to a 3-ml Pyrex test tube. The tubes were held in an ice-water bath until all enzyme transfers were completed, then they were removed to a water bath held at 23 C. After a 30 min preincubation period, the tubes were returned to the ice-water bath and then immediately assayed for enzyme activity. A sample of the preincubation mixture (10 µl, 0.12 µg protein) was added to 1 ml of a G6PDH reaction mixture containing 1.0 mM nicotinamide adenine dinucleotide phosphate ($NADP^+$) and 1.0 mM glucose-6-phosphate (G-6-P) in 0.05 M Tris-HCl buffer (pH 8.1). The maximum velocity was determined by following the production of NADPH at 23 C on a Gilford Model 240 spectrophotometer and recorder (Gilford Instrument Lab, Inc.). The percentage inhibition was calculated by comparison to the rate obtained with control enzyme which was preincubated in diluent in the absence of ARM.

A visual colorimetric assay of G6PDH (Kit No. 400, Sigma Chemical Co.) was adapted for determination of inhibition by ARM. The preincubation conditions for the enzyme were the same as described for the spectrophotometric assay. For the enzyme assay a 10 µl sample of the preincubation mixture containing 0.12 µg protein was transferred to 0.5 ml of a G6PDH reaction mixture containing 0.13 mM $NADP^+$, 5.0 mM G6P, 0.5 mM 2,6 dichlorophenol-phenazine methosulfate in 0.3 M Tris-HCl buffer (pH 8.5). After adding one ml of distilled water the surface of the reaction mixture was covered with a film of mineral oil (Sigma Chemical Co.). The tubes were then incubated at 23 C until the blue dye was reduced to its colorless form. The time required for the blue color to disappear was recorded and compared to the time required by control enzyme incubated in diluent containing no ARM.

RESULTS

The effect of preincubation time on the inhibition of G6PDH by two multiple concentrations of ARM in soft-water diluent is shown in Fig. 1. The inhibition was not linear with time but rather showed saturation kinetics after about 20 min of incubation. The

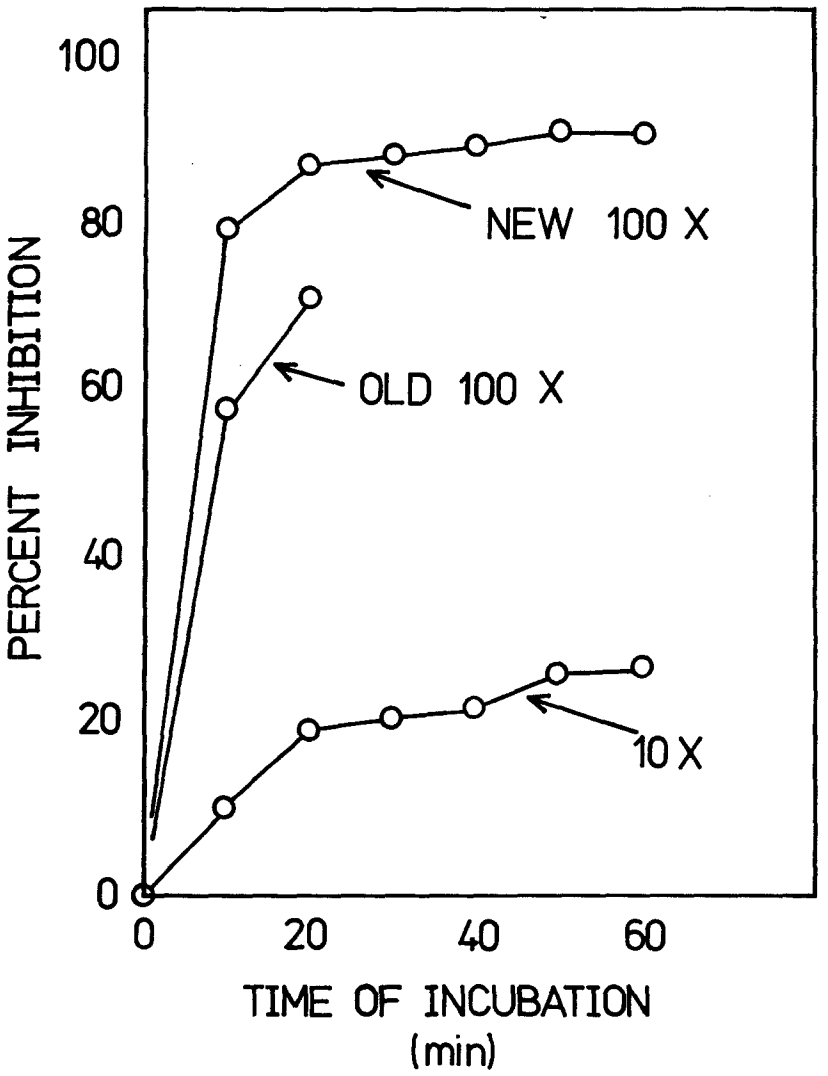

Figure 1. The effect of preincubation time on G6PDH inhibition by 10 and 100X ARM.

inhibition also was found to be dependent upon the concentration of ARM. Preincubation of the enzyme for 30 min in the presence of 100X ARM resulted in 87% inhibition, while preincubation in 10X ARM showed 20% inhibition after 30 min. Although most of the inhibition by the ARM was completed after about 20 min, a slight increase of inhibition did occur over the remainder of the 60 min incubation period.

We also tested the effect of storage of the ARM on the inhibition of G6PDH. A sample of 100X ARM stored for 6 weeks at 4 C showed no significant loss of inhibition when compared to a freshly prepared solution. Likewise, the maximum inhibition of the enzyme by the stored ARM occurred after 20 min of preincubation.

The inhibition of enzyme activity by various concentrations of ARM in both freshwater and seawater is shown in Table 1. With freshwater as the diluent, the

TABLE 1

Inhibition of G6PDH activity by various multiple concentrations of ARM after 30 min preincubation. Standard deviations were less than 20%.

	Percent Inhibition			
	Freshwater		Seawater	
Concentration of ARM	Spectrophotometric Assay	Visual Assay	Spectrophotometric Assay	Visual Assay
1X	4	24	8	0
2X	39	36	12	4
5X	36	46	19	8
10X	56	40	31	38
100X	87	84	66	58

susceptibility of the enzyme to 100X ARM resulted in 87% inhibition, whereas in seawater the inhibition was decreased to 66%. However, overall inhibition kinetics were the same for both diluents. The visual assay, though less quantitative than the spectrophotometric assay, showed a remarkably similar inhibition curve. The visual assay requires only a few reagents and could easily be performed in the field where a spectrophotometer is not available.

DISCUSSION

The assay procedure described in this report offers a simple and inexpensive method for biological assessment of environmental contaminants. Further testing of

actual industrial effluents on a complete profile of different enzymes is required in order to fully evaluate the method (these tests are now in progress). The present results show that certain preliminary steps must be done in order to define a set of assay conditions which are a valid measure of enzyme inhibition. For example, a study of the inhibition kinetics with time of preincubation in the presence of the toxicant must be completed for each new effluent. Different concentrations of the toxicant can then be tested over the linear part of the curve, or as is the case of ARM, at the preincubation time required to reach a maximum inhibition. In our preliminary experiments of the effect of ARM on G6PDH activity, a preincubation time of 5 min was used giving highly variable inhibition. A study of the preincubation conditions showed a rapid increase in inhibition over the first 20 min (Fig. 1); thus, a small error in the preincubation time resulted in high variability in the calculated percentage inhibition. Extending the time for preincubation considerably reduced this source of error.

In comparing the results of the enzyme inhibition (measured spectrophotometrically) to bioassay results for freshwater and marine invertebrates (BUIKEMA unpublisted; Table 2), significant enzyme inhibition occurred at 2X ARM, a concentration which was between the LC-50 values obtained for invertebrates and fish. These results suggest that enzyme inhibition may be a viable screening tool for refinery effluents.

TABLE 2

Comparison of G6PDH inhibition and 24 hr LC-50 values for selected invertebrates and fish tested with the ARM.

Diluent	Maximum percent Inhibition of G6PDH 2X ARM*	10X ARM	24 hr LC-50* Invertebrate	Fish
Soft freshwater	39%	56%	Waterflea 0.33X	Bluegill 7.4X
9 ppt salinity	12%	31%	Grass Shrimp 0.25-0.38X†	Mummichog 3.2-10.0X†

*Values are in multiples or fractions of the ARM formulation.
†Varies with salinity and source of the organisms.

The animal bioassays currently being used throughout the industry to assess the toxicity of effluents require considerable time, space, supplies, and personnel. Simplification of biological monitoring techniques can greatly reduce these costs. Because the toxic actions observed in screening organisms are often due to metabolic imbalances, the effect of an effluent on enzymes may constitute a valid bioassay. In addition, enzyme assays require inexpensive reagents, little space, and only minutes to complete.

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