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HUMAN BREAST SKIN TISSUE EXPOSED TO SULFUR MUSTARD (HD) MONITORED BY ^{31}P-NMR SPECTROSCOPY

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ABSTRACT—^{31}P-NMR spectra of human breast skin tissue were acquired to characterize changes in the phosphorus profile before and after exposure to sulfur mustard (HD). The tissue was rolled onto a plastic spindle, sewn in place, and inserted into an NMR tube where it was continuously perifused with phosphate-free, oxygenated culture medium. The perifusion system consisted of a pair of peristaltic pumps, an autoclavable rubber stopper, and 0.5 mm i.d. Teflon tubing through which the culture medium could be withdrawn from its original sterile container. ^{31}P-NMR spectra of control tissue were recorded for 1.5–2.0 h, the skin sample was exposed to HD (1.0 mM aqueous solution) for one h, and the spectra were again acquired. Following HD exposure, changes in the ^{31}P signals indicated changes in both intracellular pH and phosphate metabolism and/or membrane permeability. The presence of inorganic phosphate, phosphoethanolamine, and sugar phosphate in the spent perifusion buffer may be a good diagnostic indication of skin damage, whether mechanical or chemical. In several cases, microblisters appeared on the exposed skin surfaces about 12–24 h after exposure, and the epidermis could be easily detached from the rest of the dermis using tweezers.

The compound bis(2-chloroethyl)sulfide (sulfur mustard or mustard gas, HD) is a chemical warfare vesicant agent that causes severe lesions to the eyes, the respiratory tract, and the skin. This blistering agent remains a continuing threat to military and civilian populations. Sulfur mustard is most effectively employed against untrained or unprotected targets. Civilian sites are especially vulnerable.

Cellular damage due to sulfur mustard exposure occurs through alkylation reactions via a reactive episulfonium ion (Roberts and Warwick, 1963), leading to changes in phosphate metabolism. ^{31}P-NMR spectroscopy has been used extensively to monitor changes in the concentrations of phosphorus metabolites and the intracellular pH in perfused organs, such as muscle, in response to various interventions (Brindle et al., 1988). This NMR technique is the method of choice for studying the phosphorus metabolism of intact systems (Gadian, 1982). This communication describes the application of the ^{31}P-NMR technique to studies of sulfur mustard induced damage in excised human breast skin tissue. Our purpose was to use the ^{31}P-NMR technique to characterize the polyphosphate content of human skin tissues before and after sulfur mustard exposure, and to investigate various other changes in skin tissue metabolism after exposure, including intracellular pH regulation.

MATERIALS AND METHODS

^{31}P-NMR spectra were acquired using a Varian XL-300 spectrometer (Palo Alto, California) equipped with a broadband switchable, 16 mm, variable temperature probe operating at 121.4 MHz and set to 37°C. The pulse width was 53.5 μsec (70°), and the delay time was 2 sec. Chemical shifts are reported with glycerophosphocholine (GPC) at 0.49 parts-per-million (ppm), which has become a standard in recent literature (Daly et al., 1988). Chemical shifts for the compounds of interest were measured relative to GPC (internal reference) at pH 7.0 in standard solutions of similar ionic strength (~340–360 mOsmol/kg H_2O) to that of the perifusion medium (290 mOsmol/kg H_2O). These chemical shifts were in good agreement with those in the literature taken under similar physiological conditions (Lyon et al., 1986; Daly et al., 1987). The measured chemical shifts near pH 7 of different phosphorus compounds known to be present in skin tissue are summarized in Table 1.

Normal human breast skin tissue (~2–4 g, ~2.5 cm × 5 cm, fresh or frozen, National Disease Research Interchange, Philadelphia, Pennsylvania) was rinsed with phosphate-free culture medium, rolled onto a plastic spindle, and sewn in place. The spindle insert was a pair of plastic disks connected with a rigid, plastic tube. The disks were machined with outlet and inlet openings for 0.5 mm i.d. Teflon® tubing and fitted with o-rings to hold the skin and medium in the region of the receiver coil (Fig. 1). The deuterium lock was obtained by filling the microcell insert of the plastic spindle with deuterium oxide (D_2O having 99.9% isotopic purity, Sigma Chemical Co., St. Louis, Missouri) as illustrated in Fig. 1.

The perifusion apparatus consisted of an autoclavable rubber stopper equipped with glass tubing and 2.5 mm i.d. Teflon® tubing through which the culture medium could be withdrawn from its original sterile container (Fig. 2). Two peristaltic pumps

(Masterflex™, Cole-Parmer Instruments, Vernon Hills, Illinois) carried medium to and from the NMR insert. The Teflon® tubing could be easily removed from the pumps enabling the entire system to be moved to a hood during the introduction of sulfur mustard. All of the components are commercially available except the plastic spindle, which was fabricated (Reed Plastics, Rockville, Maryland). The tissue was perifused at a flow rate of 2.5 ml/min with oxygenated culture medium free of inorganic phosphate (modified MCDB 153, Clonetics, San Diego, California). The culture medium was kept under a 95% O_2/5% CO_2 environment via inlet and outlet ports in the stopper. To allow temperature equilibration, the skin sample on the spindle was placed in an NMR tube and perifused in the probe at 37°C for 15 min. Control spectra were then recorded for two h. The spindle holding the skin was immersed in sulfur mustard solution (1.0 mM, 40 ml) for one h, returned to the NMR tube, and spectra were again acquired while the sample was being perifused as before.

RESULTS

Fig. 3A shows a two-h spectrum of normal breast skin tissue from a 30-year-old Caucasian female. Chemical shifts were determined relative to GPC in aqueous solution at pH 7.00 (external reference, 0.49 ppm). The signals are assigned (from downfield) as glucose-6-phosphate (G-6-P)/phosphoethanolamine (PE), 3.96 ppm; sugar phosphate (SP), 3.22 ppm; inorganic phosphate (Pi), 1.90 ppm; GPC, 0.38 ppm; and nicotinamide adenine dinucleotide, reduced form (NADH), −10.78 ppm. No signals clearly attributable to ATP or ADP appeared. The spectrum for the same skin sample after a one-h aqueous sulfur mustard exposure is shown in Fig. 3B. The phosphorus signals remaining after the exposure can be assigned to G-6-P/PE, 3.86 ppm; SP, 3.08 ppm; and Pi, 1.75 ppm. The most interesting result was that microblisters formed on the epidermis of some skin samples about 12–24 h after the one-h exposure to sulfur mustard. Blister formation did not occur when the skin was soaked without mustard in deionized water or in isotonic buffer. The recorded ^{31}P-NMR spectrum (data not shown) of normal breast skin tissue from a 19-year-old Caucasian female exhibited several signals that were assigned (from downfield) as PE, 4.17 ppm; Pi, 2.41 ppm; GPC, 0.25 ppm; and phosphoenolpyruvate (PEP), −0.04 ppm. No signals attributable to ATP and ADP were observed. The remaining ^{31}P-NMR signals of the same skin sample after a one-h exposure to 1.0 mM sulfur mustard can be assigned to PE, 4.01 ppm and Pi, 2.28 ppm. In these two spectra of human skin tissue samples, Pi decreased by at least two-fold after sulfur mustard exposure. Furthermore, the Pi peaks in the NMR spectra of both tissues are shifted upfield after sulfur mustard exposure indicating a pH decrease. These are responses that were expected in the presence of sulfur mustard due to the halo acids formed by its hydrolysis (Shasteen and Reed, 1983).

One experiment made use of breast skin tissue from a 74-year-old, Caucasian female. This skin sample contained a lot of fat tissue and had sustained mechanical injury prior to the experiment as evidenced by bruising. The signals in the control spectrum were attributed to adenosine monophosphate (AMP)/SP, 4.15 ppm; Pi, 2.50 ppm; and GPC, 0.40 ppm. A spectrum of the same skin after a one-hour exposure to 1.0 mM sulfur mustard showed only the Pi and SP signals remaining with their intensities reduced, while a spectrum of the perifusate collected after exposure to the mustard showed that SP and Pi had been lost (data not shown). A spectrum of the perifusion buffer collected during the control experiment prior to the mustard exposure showed more loss of Pi from the skin than the post exposure buffer showed. Results from five other skin experiments also showed a measurable loss of phosphorus compounds to the perfusate both before and after exposure of the skin to sulfur mustard, though these skin samples lost less of their phosphorus content during the control experiment than did the sample from the 74-year-old female. The dermis in all samples had sustained mechanical injury resulting from excision. Whether the loss was from the epidermis in addition to the dermis is uncertain. The sample from the 74-year-old female showed no blistering, but the previously described skin samples from the 30- and 19-year old females showed small blisters on the epidermis about 12–24 h after mustard exposure. Blistering seemed to be directly related to the state of health of the skin; that is, the more resilient, healthier looking skin seemed most susceptible to the blistering (Papirmeister, 1993).

It is interesting to note that the NMR spectra of human breast skin tissue showed no ATP or ADP signals. Unfrozen skin was

TABLE 1. Chemical shifts of biochemically important phosphorus compounds in 340–360 mOsmol/kg H_2O solution at 37°C relative to glycerophosphocholine (GPC). GPC is the internal reference with resonance set to 0.49 ppm.[1]

pH	Signal[2]	Chemical Shift (ppm)	Δ from GPC
7.00	GPC	0.49	0.00
7.00	PCr	−2.45	−2.94
6.99	PE	4.30	3.81
6.99	NADH	−10.58	−11.1
7.00	β-ADP	−6.19	−6.68
7.00	α-ADP	−9.94	−10.43
7.00	AMP	3.85	3.36
7.00	G-1-P	2.53	2.04
6.79	Pi	1.99	1.50
6.96	Pi	2.23	1.74
7.00	Pi	2.31	1.82
7.42	Pi	2.79	2.30
7.00	γ-ATP	−5.00	−5.49
7.00	α-ATP	−10.19	−10.68
7.00	β-ATP	−19.60	−20.09
7.00	G-6-P	4.56	4.07
7.00	PEP	−0.09	−0.58
7.00	PEP	−0.04	−0.53
7.42	PEP	0.16	−0.33
7.01	G-1, 6-diP (6P)	4.85	4.36
7.01	G-1, 6-diP (1P)	2.74	2.25
7.00	GPE	0.99	0.50

[1] ppm is parts-per-million.

[2] PCr is phosphocreatine, PE is phosphoethanolamine, NADH is nicotinamide adenine dinucleotide (reduced), ADP is adenosine diphosphate; AMP is adenosine monophosphate, G-1-P is glucose-1-phosphate, Pi is inorganic phosphate, ATP is adenosine triphosphate, G-6-P is glucose-6-phosphate, PEP is phosphoenolpyruvate; G-1,6-diP is glucose-1,6-diphosphate, and GPE is glycerophosphoethanolamine.

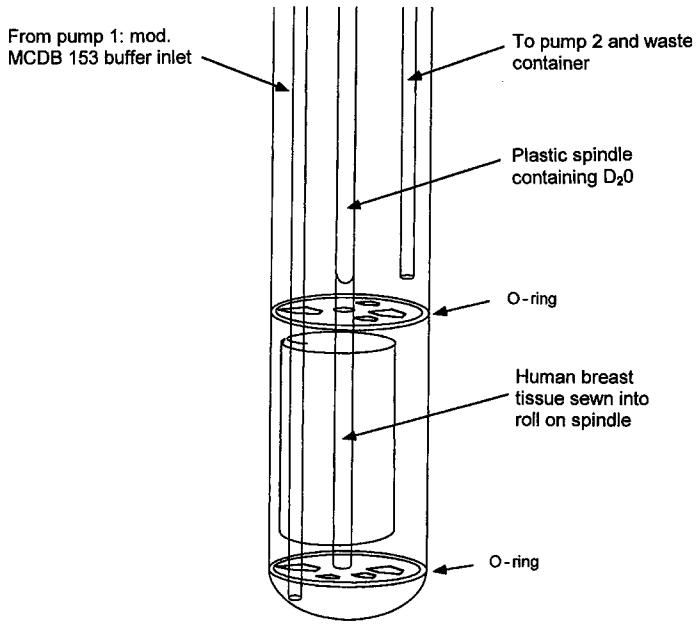

FIG. 1. NMR tube with spindle insert for perifusing mounted skin tissue. Apparatus modified from McCreery et al., 1989.

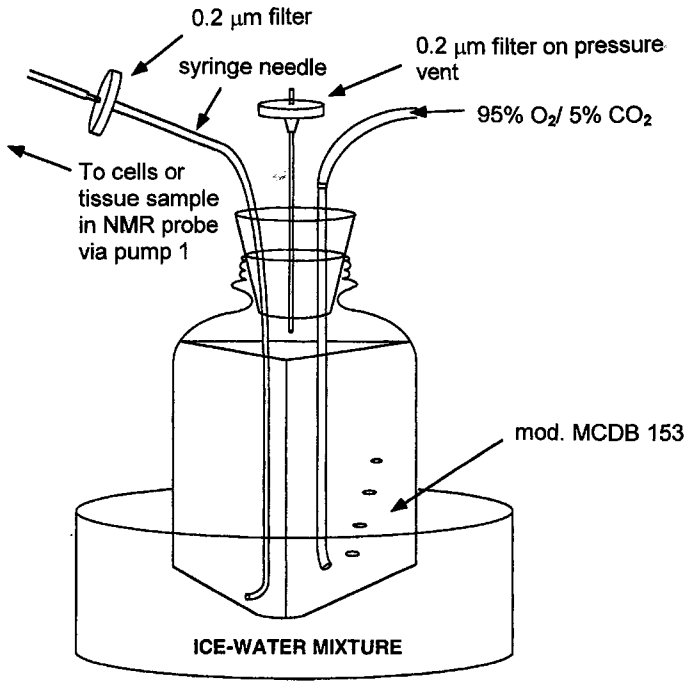

FIG. 2. Apparatus for perifusing skin tissue allows medium to be pumped from original, sterile container.

used to help avoid hydrolysis or loss of ATP, ADP, and Pi from the tissue due to damage by the freeze-thaw process. The skin was received fresh packed in water ice. However, due to shipping, several hours had elapsed since excision. The continuous perifusion with phosphate-free medium before and during spectral acquisitions also may have encouraged ATP and ADP hydrolysis. Control spectra were recorded in which tissue samples were preincubated with the phosphate-free culture medium for a one-h to determine whether the loss of the phosphorus signals was a function of time or of the perifusion process. No changes were observed as a function of preincubation time.

DISCUSSION

In previous experiments in this laboratory, both ATP and ADP signals were present in samples of pig skin and of normal human epidermal keratinocyte (NHEK) cells (McCreery et al., 1989; Bonicamp et al., 1995; Bonicamp et al., 1996). These results indicate that it is possible to obtain a phosphorus profile directly from excised pig skin using ^{31}P NMR (McCreery et al., 1989). Extracts of pig skin, which were lyophilized, stored at −78°C, and then dissolved (1.5 g of the powder in 10 ml of D_2O) show three peaks attributable to ATP and ADP (Bonicamp et al., 1995). The extent by which the peak area of either of the two downfield peaks exceeds that of the upfield peak allows calculation of the ADP:ATP ratio. Along with the peak areas of AMP and Pi signals, these measurements provide an index of the metabolic activity. Fig. 4 shows the spectrum acquired from a solution of the fresh, milled pig skin extracts. The fresh pig skin was pulverized in a ball mill and extracted immediately with perchloric acid, then neutralized before storing at low temperature for NMR analysis. The ATP and ADP signals are clearly identifiable in the spectrum.

The spectra in Fig. 3 are of "solid" tissue samples in which chemical reactions are occurring. These tissue samples were held stationary in the receiver coil where the phosphorus nuclei experience a variety of magnetic environments. Thus the lines in the Fig. 3 spectra are broad and the resolution suffers. The spectrum in Fig. 4 is from a solution in which the biochemical processes have been quenched by chemical treatment and freeze-drying. The tube containing the solution is spun within the receiver coil to average out inhomogeneities of the magnetic field. Useful spectral data can be acquired for hours under these conditions yielding superior resolution. The relative signal heights for Pi and ATP are about 3:1 in Fig. 4. If ATP is present in any of the breast skin tissue samples the amount is quite low. In spite of line broadening, the ATP resonance would be visible in Fig 3 at a signal intensity one third that of Pi. There is no dilution effect resulting from application of the sulfur mustard to the tissue samples. The skin occupies a constant volume throughout the experiment. The sample, spindle, and perfusate occupy a constant 10-ml volume in an NMR tube both before and after mustard exposure. The only dilution effect is that resulting as phosphorus compounds are lost to the buffer during perifusion.

Figure 5A shows a two-hour spectrum of normal pig belly skin tissue, excised and refrigerated overnight, and then perifused with oxygenated, phosphate-free culture medium during NMR analysis (unpublished results from this laboratory). The signals are assigned from downfield as G-6-P/PE, Pi, and NADH. Figure 5B shows a spectrum of the same pig skin sample after a one-h exposure to 1.0 mM aqueous sulfur mustard solution. The phosphorus signals remaining can be assigned to G-6-P/PE and Pi. The Pi signal is shifted 0.34 ppm upfield as a result of the mustard exposure. The spectrum of normal pig skin is slightly different from the spectrum of normal human breast skin tissue. Spectra reported by others for samples of mammalian tissues or cells resemble the spectra obtained in these studies (Barany and Glonek, 1984).

The spectra shown in Figs. 3 and 5 lack signals from ATP and ADP, which are standard markers of tissue viability. The ^{31}P-NMR spectrum of extracted fresh tissue (Fig. 4) is an example of what should be expected from perifused tissue. The reason for

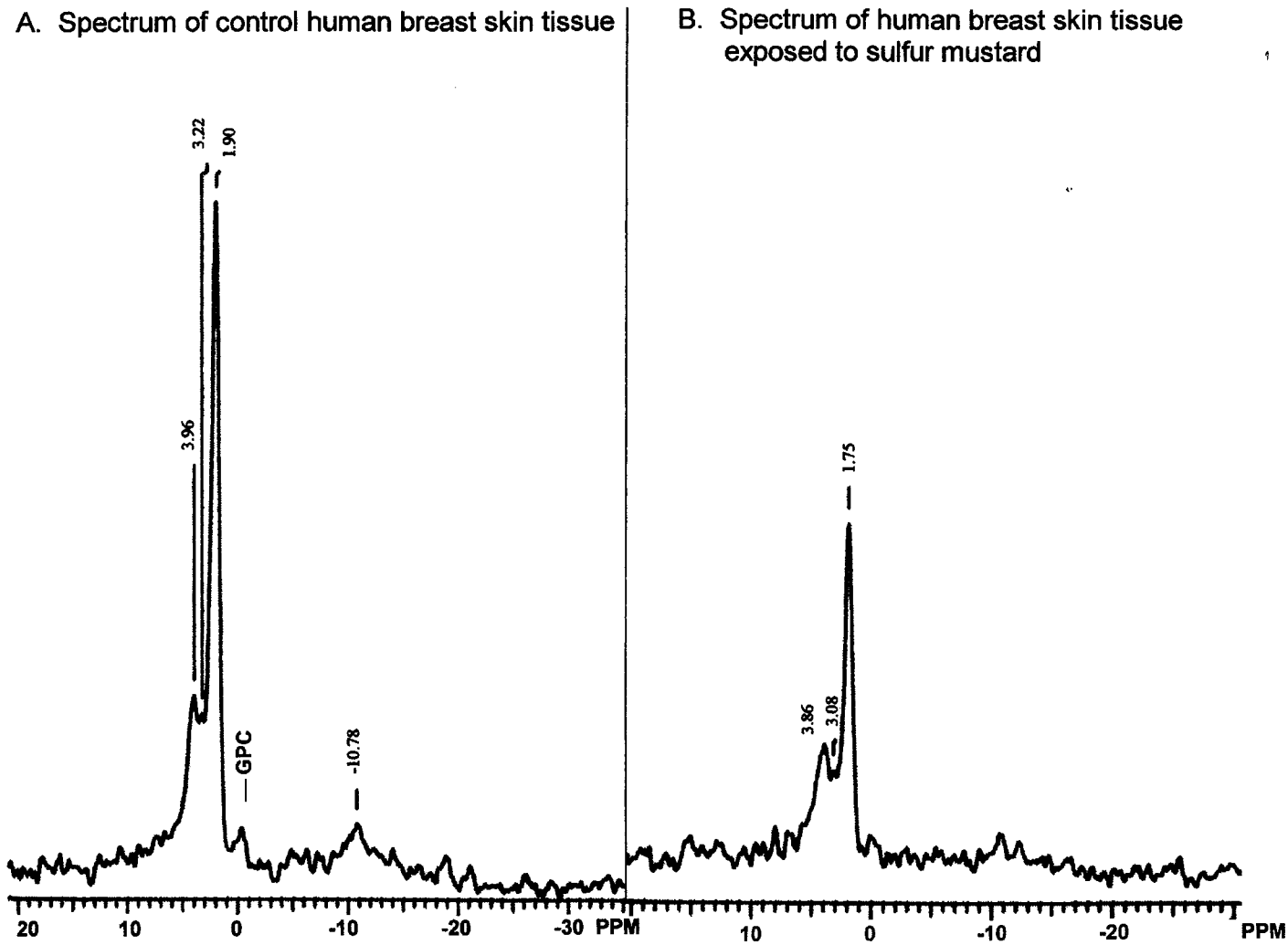

FIG. 3. A. Two-h ^{31}P-NMR spectrum with 2 sec repetition time (3600 scans), pulse width 53.5 μsec (70° pulse angle), of normal human breast skin tissue at 37°C, pH 7.0; tissue was from a 30-year-old Caucasian female. B. Spectrum of same tissue after a one-h exposure to 1.0 mM aqueous sulfur mustard.

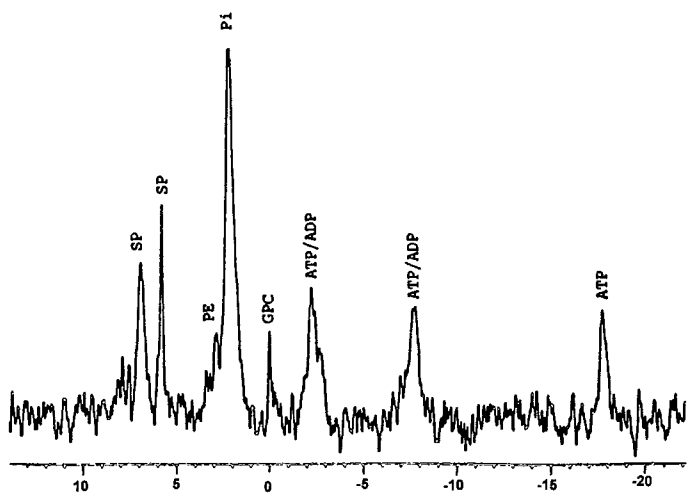

FIG. 4. Twelve-h ^{31}P-NMR spectrum with 1.25 sec repetition time (25,000 scans), of a pig skin extract solution at pH 8.3, 25°C (Bonicamp et al., 1995). Chemical shifts are relative to GPC (internal reference, 0.00 ppm) and are uncorrected.

the lack of viability for the human skin and pig skin could be that several hours had elapsed between excision and spectrum acquisition.

CONCLUSION

The ^{31}P-NMR spectra of nonexposed and of exposed human breast skin tissues show differences. The inorganic phosphate peaks of some of the exposed spectra are shifted upfield somewhat, indicating a pH decrease within the exposed tissues. This result was expected since bis(2-haloethyl)sulfides are known to produce halo acids upon hydrolysis. Some phosphorus signals are missing from spectra of the exposed samples. The changes in the chemical shifts as well as the loss in intensities of phosphorus signals following mustard exposure are indicative of a change in phosphate metabolism and/or a change in the permeability of the cell membrane. More experiments including those using fibroblasts and epidermal keratinocyte (NHEK) cells are needed for an understanding of these changes. Finally, we have shown that the presence of Pi, PE, and sugar phosphate in the perifusion buffer may be a good diagnostic indicator of skin dam-

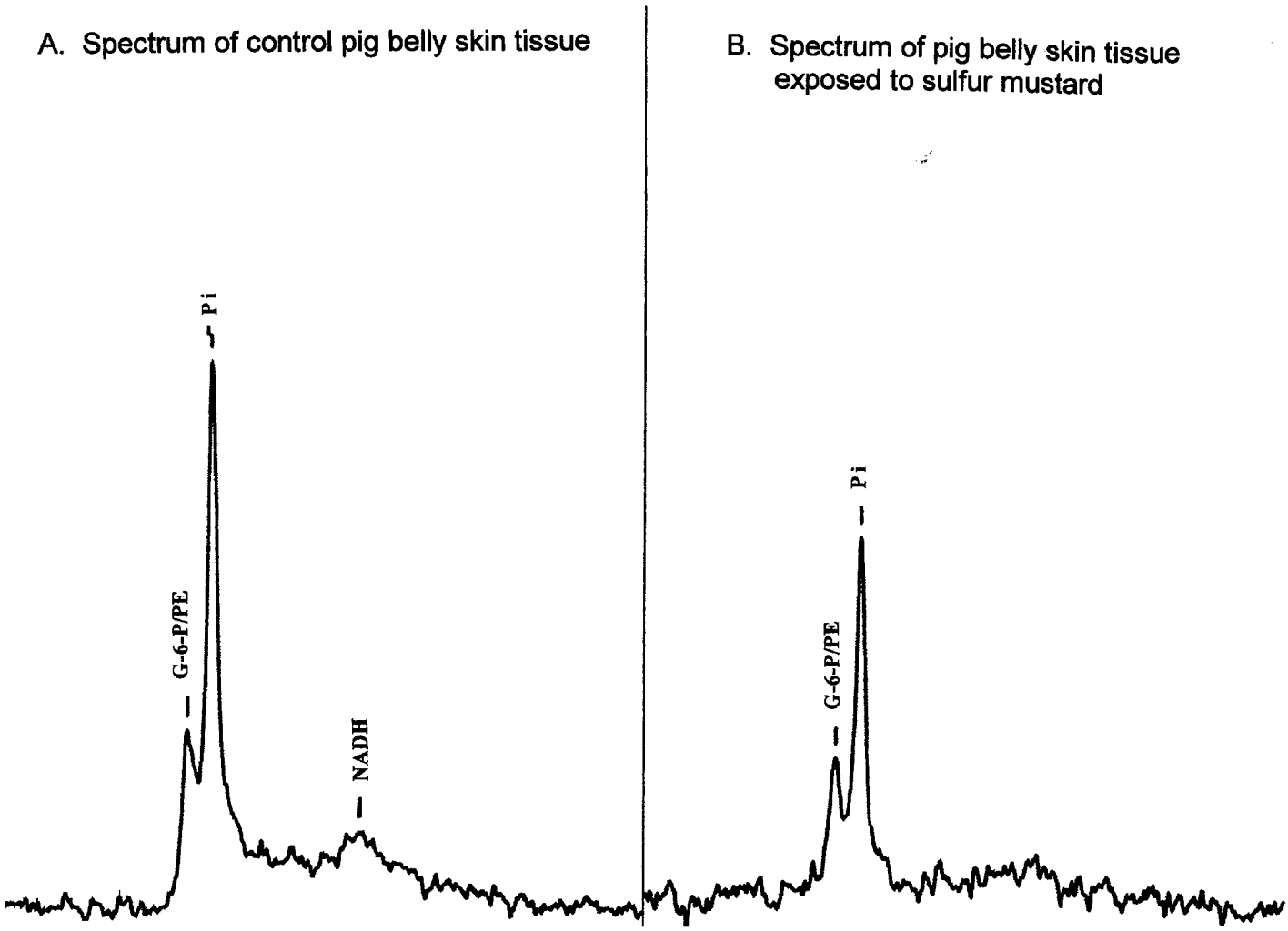

FIG. 5. A. Two-h ^{31}P-NMR spectrum with 2 sec repetition time (3600 scans), pulse width 53.5 μsec (70° pulse angle), of normal pig belly skin tissue at 37°C. B. Spectrum of same tissue after a one-h exposure to 1.0 mM aqueous sulfur mustard.

age, whether mechanical or chemical. This should prove useful in experiments where a wash of the skin, after some perturbation, is examined for ^{31}P-NMR signals.

The eventual goal is to elucidate the biochemical mechanisms that distinguish human pathological processes so that a differential diagnosis can be made based upon direct in vivo evaluation of the tissue exposed to sulfur mustard. In addition, the noninvasive and harmless character of the NMR method may permit continual evaluation of subsequent therapeutic intervention.

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