Some Factors Associated with the Ultrasonic Nebulization of Proteins

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Ultrasonic nebulization of lactate dehydrogenase (LDH) was investigated using a DeVilbiss "Aerosonic" nebulizer. The enzyme (8ml, 0.025mg/ml Na_2HPO_4, pH 7.0) was completely inactivated after 20 minutes of operation. However, the inactivation profile observed during ultrasonic nebulization was different from that previously observed using air-jet nebulization. At least two mechanisms are involved, one associated with heating and the other with aerosol production. By preventing heating of the nebulizer fluid during operation, the denaturation profile was dramatically altered. By additionally including 0.01% w/v Tween 80 or 1%w/v PEG 8000, almost all activity was retained. Similar results were obtained by preventing aerosol production and heating. However, 100% of activity was lost when heating was allowed to occur without aerosol formation. The results demonstrate that cooling in conjunction with a surfactant is one approach that could be used to stabilize proteins to ultrasonic nebulization. However, cooling also significantly reduced solute output from the nebulizer. When operated at 10°C output was negligible. At 50 °C the output was 5x greater than that found at room temperature. The median droplet size (μm) was not significantly influenced by the operating temperature of the nebulizer fluid (3.6±0.4, 21°C; 3.9±0.2, 50°C, p=NS (n=6)) although the size distribution was noted to increase at the higher temperature.

KEY WORDS: aerosol; lactate dehydrogenase; nebulizers; proteins; ultrasonic nebulization; stability.

INTRODUCTION

A number of proteins have been delivered to the lung via inhalation aerosol (1,2). Pulmozyme is one example that is now used clinically and is delivered by air-jet nebulizer (3). As an alternative, a variety of ultrasonic nebulizers are available for inhalation therapy but the effect of ultrasonic nebulization on proteins has not been thoroughly investigated. In addition, despite the commercial use of these devices, the fundamental mechanism of ultrasonic nebulization is poorly understood (4–7). Consequently, it is difficult to determine what factors might be responsible for any observed destabilization of proteins. Most theoretical research has focused on the industrial application of ultrasonic energy for such uses as cleaning and coating. Cleaners use high power, low frequency ultrasonic waves to scrub materials by cavitation and usually no aerosol is produced. Ultrasonic nozzles produce aerosol by passing a continuous liquid feed through a vibrating 'horn' that amplifies the mechanical oscillations generated by a piezoelectric transducer (4,8). The frequency of the resultant capillary wave formation at the atomizing surface dictates the droplet size (9). Medical nebulizers differ in that the energy generated from the piezoelectric crystal is coupled and focused directly through the nebulizer solution. This results in the production of a fountain, due to pressure radiation, from which aerosol is produced near the base of the geysor. The droplet size range produced by these devices is of an order of magnitude smaller than used for most industrial applications (10,11) and cannot be predicted from the capillary wave theory alone (5). There is evidence in fact, that both cavitation and capillary wave effects occur in medical nebulizers (5,12). The capillary wave theory suggests that droplets are released from Taylor instability arising at wave crests on the surface of the sonicated liquid (9). The cavitation theory postulates that implosions of tiny bubbles close to the surface of the liquid generate a disturbance in the surrounding fluid that is of sufficient violence to generate a spray (13). It is now thought that the cavitation disturbances interact with the capillary waves and form droplets by the same mechanism (14).

Most of the energy transduced through the nebulizer solution is dissipated as heat (10). This does not normally pose a stability problem for current inhaled drugs (15) but the heating could be detrimental to thermolabile formulations such as proteins and can cause odor changes in antibiotic solutions (16). It has recently been noted by Cipolla et al (17) that rhDNase can be aggregated by ultrasonic nebulization if the melting transition temperature of the protein is approached or exceeded in solution. The heating however, is merely a by-product of ultrasonic aerosol generation and breakdown of formulations may be the cumulative effect of heat, surface denaturation, cavitation and even the direct pressure effects of ultrasonic radiation. Wigley et al. (18) also have noted that insulin was inactivated by ultrasonic nebulization but did not investigate the problem further. Alpha-interferon has been studied by Sato et al. (19) using two ultrasonic nebulizers operating at 80 and 160kHz. They showed that the protein was inactivated by the lower frequency but not at the higher and suggest that 'visible' or 'degassing' cavitation was responsible for the loss of interferon activity. Two nebulizers currently in use in the United States are the DeVilbiss "Aerosonic" and the Mountain Medical "Microstat." Both operate in the mHz range (Aerosonic–2.2mHz; Microstat–1.8mHz) at greater than 10x the frequency of the nebulizers studied by Sato et al. (19). The purpose of this study was to isolate and individually examine the factors that would influence the stability of proteins during high frequency ultrasonic nebulization. We then hoped to find means of reducing the detrimental effects of the process. Lactate dehydrogenase (LDH) was employed as the model protein and solutions were nebulized using the DeVilbiss "Aerosonic" nebulizer. Tween 80 and polyethylene glycol were added to solutions as potential stabilizers. The influence of cooling and aerosol production on LDH activity were also examined.

MATERIALS AND METHODS

Enzyme Preparation

Lactate Dehydrogenase isolated from rabbit muscle was purchased from ICN (Lot #'s 41628 and 42337, Irvine, CA)

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in a stabilizing solution of 65% saturated ammonium sulfate, pH 7.2. The protein was dialyzed and then diluted to 0.025mg/ml before experiments as discussed previously (20).

Sample Analysis

Enzyme assays were completed using a Beckman DU 650 UV spectrophotometer with a temperature controlled six cuvette holder as described previously (20). Briefly, the enzyme samples were diluted 10 fold before conducting the assay. Next, 50 µl of the diluted enzyme sample was added (and mixed) into a disposable cuvette containing 1.025 ml of 23 mM Tris, 93 mM KCL, 2 mM pyruvate, and 0.15 mM NADH, reduced form (pH 7.5). Enzyme activity was determined by measuring the change in A_{340} over one minute. Samples at the start and end of nebulization were also analyzed for light scattering by visible spectrophotometry at a wavelength of 340nm. In a separate experiment, 200µl samples were removed during nebulization and injected onto a Biosep SEC 2000 column (300 x 7.8 mm, Phenomenex, Torrance, CA) incorporated into a Waters (Bedford, MA) liquid chromatography system with a 490 Multiwavelength detector. The mobile phase was 0.1 M Na Phosphate, pH 6.9 and a flowrate of 1 mL/min was employed. Peak elution was monitored spectrophotometrically at a wavelength of 220 nm. Samples were held at 5°C in a Waters® 717 auto sampler.

Ultrasonic Nebulization

All nebulization experiments were completed using a DeVilbiss "Aerosonic" ultrasonic nebulizer (Model 5000; DeVilbiss, Somerset, PA). This nebulizer will operate continuously for approximately 40 seconds and then will automatically cutout. Continuous nebulization was achieved by pushing the restart button just before the operating cycle of the nebulizer was completed. The starting reservoir volume used for all studies was 8 mL. Samples of 50µl were removed from the reservoir fluid at regular intervals during nebulization for subsequent assay. The operation of the nebulizer was not halted when samples were removed. The temperature was monitored by a digital thermometer (Model 8402-20; Fisher Sci., Pittsburgh, PA) with a probe (Model 423 YSI probe; Fisher Sci.) inserted through the aerosol outlet of the nebulizer (Fig 1). Initial experiments were completed by allowing the nebulizer to operate normally with the exception that aerosol was cycled within the nebulizer. That is, there was no air flow through the nebulizer.

Effect of Heat on LDH Activity

Since ultrasonic energy will result in rapid heating of nebulizer solutions, the effect of heat on the stability of LDH was studied. The nebulizer was modified to accommodate a coil of stainless steel tubing (Fig 1). This was connected to a water bath (Lauda RC6, Brinkmann, Westbury, NY) containing a 1:lv/v mixture of water and refrigerant. By adjusting the water bath temperature appropriately, it was possible to maintain the temperature of the nebulizing fluid to within 1 or 2 degrees at the site of measurement. Previous experiments (20) have shown that aerosol production or the continuous production of new surface is a dominant factor in the inactivation of LDH during air-jet nebulization. To prevent

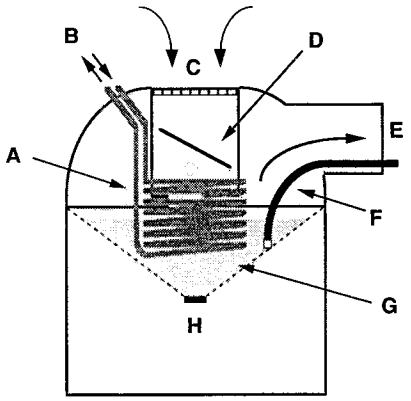

Fig 1 Description of the modified DeVilbiss ultrasonic nebulizer. (A) Stainless steel cooling coil submerged in the nebulizer solution. (B) Inlet and outlet connected to a refrigerated water bath containing coolant. (C) Nebulizer intake vent. (D) Nebulizer baffle. To prevent aerosol production a second baffle was placed lower directly in the path of the ultrasonic fountain. (E) Aerosol outlet. (F) Temperature probe. (G) Nebulizer reservoir. (H) Piezoelectric transducer (2.2mHz).

aerosol production, but otherwise allow the nebulizer to operate normally, a baffle of silicone rubber was fixed in position directly in the path of the ultrasonic fountain. This completely prevented aerosol production and enabled us to study the effects of ultrasonication on LDH in isolation of any aerosol formation. Experiments were then conducted for up to 40 minutes operating time with and without heating of the LDH solution.

Effect of Aerosol Production on LDH Activity

In these experiments aerosol production was studied at a fixed operating temperature. The nebulizer fluid was maintained at 25±2°C and the silicone baffle was removed to allow aerosol formation.

Influence of Additives on LDH Activity

It was previously noted that polyethylene glycol of various molecular weights and Tween 80 in solutions of LDH could protect the protein to the destabilizing environment of nebulization (20). We conjectured that these protectants might exhibit similar protective qualities during ultrasonic nebulization. Solutions of 1%w/v PEG 8000 and 0.01%w/v Tween 80 with LDH were nebulized in the presence and absence of heating, as described above, for up to 40 minutes.

Effects of Heating on Aerosol Solute Output

The effect of temperature on solute output was studied by operating the nebulizer while maintaining the temperature of the solution at 10, 20, 25, 30, 40 and 50°C. The solute output was collected by generating an air-throughput of 5 L/min using a vacuum pump (Model DOA P2I 7-AA, Fisher Sci. Pittsburgh, PA) and collecting the solute output over a 10 min period by first passing the aerosol through a heated (≈90°C) stainless steel column and then collecting the solute in a 90mm stainless steel filter unit (Millipore. Bedford, MA) containing a 90mm glass fiber filter (Type A/E, Gelman Sci., Ann Arbor, MI). To determine the mass output, 10µg/ml

carboxyfluorescein was added to the LDH solution. The carboxyfluorescein emerging from the nebulizer was then assayed by spectrofluorimetry as described elsewhere (21). The presence of the coil also did not interfere with the operation of the nebulizer. This was checked by allowing the nebulizer to operate in the presence and absence of the coils while collecting the output as described.

The cumulative output was also determined for the nebulizer operating with heating and at the initial temperature of 21°C. Here the solute output was collected for 2, 4, 6, 8 and 10 minutes. It was not feasible to collect the output every two minutes during operation since the time involved removing filters and cleaning all surfaces on which the aerosol deposits, would result in some cooling of the nebulizer solution. The solute concentration in the nebulizer was determined over the same 10 minute period for the above experiment. This enabled us to differentiate between increases in output due to concentration of solute in the reservoir fluid and the increase in output due to the influence of temperature on aerosol formation.

Effect of Heating on Droplet Size

The effect of heat on droplet size was studied at 20 and 50°C. The output at 10°C was too low to collect sufficient mass of material for assay. To determine any relative change in the droplet size, the 'residual' or 'back calculation' technique was used (22). The droplets were dried using the method described above but an Andersen cascade impactor was substituted for the collection unit and a diffusion dryer was added to the outlet of the heating column similar to that described before (20). A solution of 5%w/v NaCl and 10μg/ml CF was substituted for the LDH solution to ensure that the dried particles would fall within the size range of the impactor. Since the vacuum through the impactor draws 28.3 L/min a 'Y'-connector was attached between the outlet of the drier and inlet of the impactor. Flow through the nebulizer was fixed at 2L/min and monitored by a mass flow meter (Model 820; Sierra Inst., Monterey, CA) attached to the inlet of the nebulizer. Make-up air was introduced via the 'Y' connector. The relative humidity and temperature of the air emerging from the drier were checked at the end of each experimental run to ensure that the particles were being dried.

RESULTS AND DISCUSSION

Ultrasonic Nebulization

It is reasonable to expect that ultrasonic nebulization might be more damaging to proteins than air-jet nebulization. The power output from the ultrasonic device is coupled directly through the nebulizing fluid and this can generate enormous transient g-forces within the nebulizing fluid. There is even the potential to break covalent bonds (5). In addition to aerosol production, a great deal of energy is dissipated as heat. In contrast, much of the energy, stored as compressed air, utilized in air-jet nebulization, is not used to generate aerosol and simply acts as a carrier for the resultant aerosol. However, given the fundamental differences in the mechanisms of aerosol production it is perhaps not surprising to find that it is really the nature of the inactivating process and not necessarily the degree of inactivation that differs between the nebulizing techniques. This is borne out by a comparison of the inactivation curves of LDH by ultrasonic nebulization and air-jet nebulization (Fig 2). The inactivation of LDH by air-jet nebulization follows apparent first-order kinetics (19). The inactivation by ultrasonic nebulization is non-linear and a sigmoidal profile is exhibited. This suggests that more than one process of inactivation is associated with ultrasonic nebulization of LDH. The inactivation profile via ultrasonic nebulization follows the same trend as the loss of the main peak eluting from the SEC column for progressively nebulized samples (t = 5 min. 83% vs 83%; t = 10 min 53% vs 78% and t = 20 min 12% vs 32% [% activity vs % peak area]). Assuming that the main peak represented the active tetrameric species, the loss of activity appears to be a combination of aggregation and loss via the progressive appearance of a series of smaller molecular weight peaks eluting from the column. These peaks could represent monomer and dimer subunits. The production of insoluble aggregates also was indicated by an increase in light scattering at 340nm and the presence of a small pellet was observed in post-nebulized samples centrifuged for 15 min at 15,000G.

Temperature Changes During Aerosolization

Mercer et al. (10) have illustrated and mathematically described the heating curves that occur during ultrasonic nebulization. The basic process involves a constant heat input (k_0) and a first order dissipation of heat (k_1) to the surrounding environment. The change in temperature with time (ΔT) is then given by

$$\Delta T = \Delta Tm*(1-e^{-k_1*t}) \quad (1)$$

where (ΔTm) is the difference between the initial and plateau temperature (ΔTm = ratio of k_0/k_1). Fitting the observed

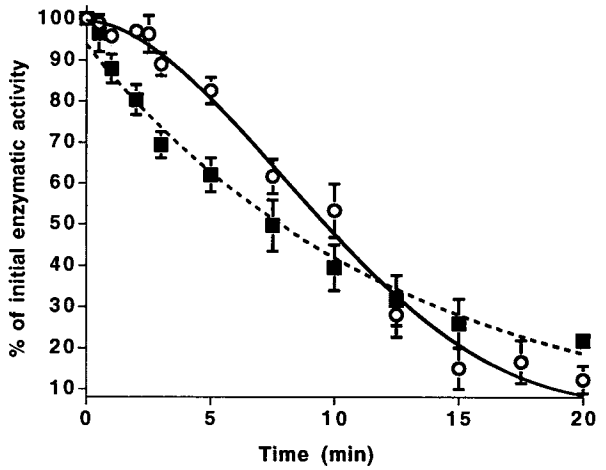

Fig 2 The inactivation of LDH by air-jet (■) and ultrasonic nebulization (○). The air-jet nebulization was performed at 40 psig in a 3-jet Collison nebulizer with an initial volume of 10ml (19). The ultrasonic nebulization was completed in the DeVilbiss "Aerosonic" nebulizer with an 8ml starting volume. An exponential decline (---) in activity via air-jet nebulization is illustrated whereas the nature of inactivation by ultrasonic nebulization (—) is sigmoidal. Error bars are the standard error of the mean (n≧5).

data over the first 15 min of normal operation of the nebulizer gave $k_1 = 1.1\pm0.2x10^{-2}$ min^{-1} and $k_0 = 4.2\pm0.5$ degC/min (n=3). However, eq 1 is not rigorously correct and only applies to static conditions where no aerosol is removed from the device. Since fluid volume is lost during normal use, the capacity of the nebulizing fluid to accommodate heat will drop. Hence, for a constant heat input and reducing volume the temperature should continue to increase and not plateau. This is offset to a varying degree by the output of aerosol which acts as an additional heat sink. Thus the temperature increase with airflow is curtailed relative to that observed with no air flow (Fig 3). The solute output during nebulization is also not constant as will be seen. In addition, a large temperature differential is quickly created between that of the fluid in the ultrasonic fountain and the surrounding fluid. The temperature ramp with the probe in the ultrasonic fountain is considerably faster than with the probe in the bulk fluid (Fig 3). Unfortunately, the probe could not be placed in the fountain 'hot spot' during experiments since it interfered with aerosol production. It was therefore offset 1 cm from center inside the nebulizer so that a comparison of the 'general' heating curves could be made for the different experimental conditions. All these factors together, make it difficult to realistically model the nebulizer heating curves. This is further complicated by the fact that the DeVilbiss nebulizer has a thermal cutoff switch that activates at ≈55 °C. Once switched off, the nebulizer fluid must cool for several minutes before nebulization can be resumed.

Effect of Heat and Aerosol Production on LDH Activity

The inactivation kinetics of LDH during ultrasonic nebulization led us to hypothesize that at least two processes of inactivation were occurring: one associated with surface denaturation (through aerosol formation) and the other due to thermal inactivation. To address the hypothesis, experiments were conducted a) in the absence of heating, b) without aerosol formation, and c) with no heating or aerosol production.

When the nebulizer is operated at 25°C, the inactivation of LDH follows linear kinetics: similar to that observed with air-jet nebulization (Fig 4). However, when the nebulizer is allowed to heat, without aerosol production, the inactivation rate is curtailed for several minutes but then increases rapidly, so that after 20 minutes little activity remains. When the nebulizer is operated so that neither aerosol production nor heating is allowed, the loss of activity is markedly reduced and only some 15% is lost over 40 minutes nebulization (Fig 4). These results support the hypothesis that heating and aerosol production are primarily responsible for the inactivation of LDH. The rapid loss of activity noted during heating alone is probably a consequence of the temperature within the ultrasonic fountain exceeding the melting transition temperature (T_m) of the LDH. The T_m of the LDH is 56°C as obtained from UV/visible spectrophotometry and the literature (24). This is well below the temperatures that can be reached in the geyser (Fig 3). Since the fluid is cycling within the nebulizer, eventually it should all be exposed to the high temperature of the fountain. When the LDH is placed in a water bath and the inactivation kinetics are monitored at 25, 55 and 60°C the loss of activity with time is rapid above the melting temperature and negligible at 25°C (Fig 5). It is also feasible that the effects of sonication might affect proteins and could denature them directly or else sensitize them to the effects of aerosolization or heating. If the 'state' of sonication can be considered like any change in the local environment, such as a change in pH, this postulate is reasonable. In the case of the LDH, the high frequency expan-

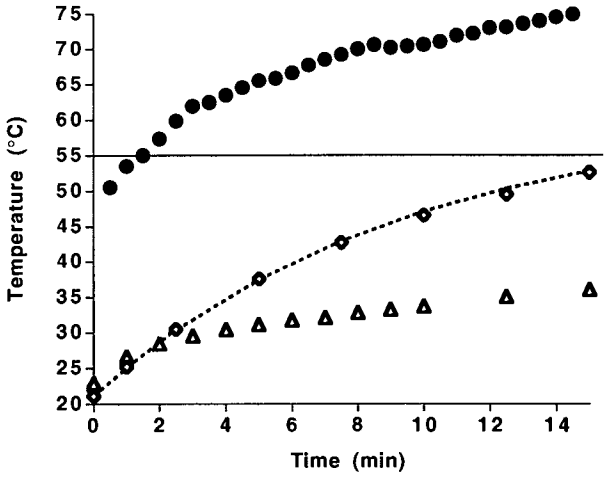

Fig 3 Temperature changes in the nebulizer solution as a function of time under different operating conditions. Temperature of the nebulizer bulk solution (◆) and within the ultrasonic fountain (●) during normal operation. The best fit (---) obtained using eq 1 is also shown for the bulk solution. Temperature of the nebulizer solution (▲) while flow of 5 L/min ambient air passes through the nebulizer. When temperatures are raised above ≈55°C the nebulizer shuts off and requires to cool to around 45°C before aerosolization can be continued.

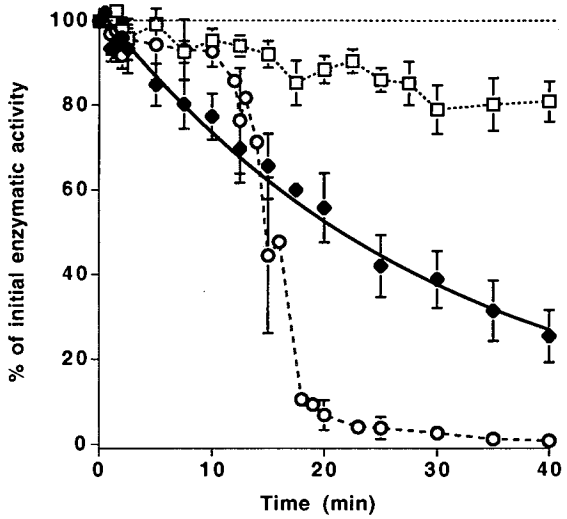

Fig 4 Isolation of the individual factors causing inactivation of LDH during ultrasonic nebulization. The effects of operating the nebulizer at a) ambient temperature (25°C; ◆) b) without aerosol production (○) and c) ambient temperature and no aerosol production (□) are shown. The bold line through ambient temperature data shows the exponential decline in activity under these conditions. When operated without aerosol production but with normal heating there is relatively little loss of activity until ≈10min (45 to 50°C). After this time activity drops rapidly. Data shown is the mean±standard deviation (n≧4).

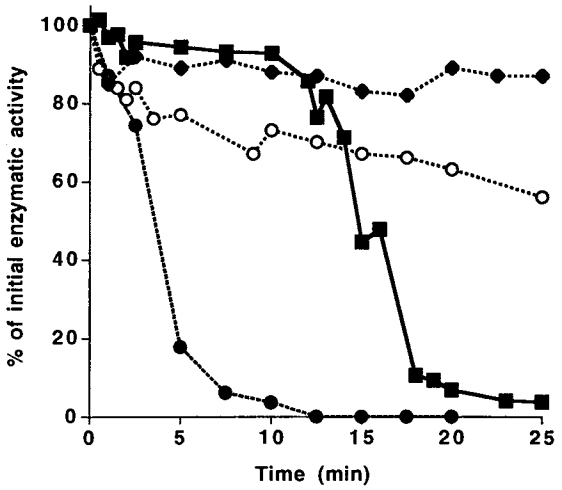

Fig 5 The effect of temperature of the LDH solution with and without ultrasonication. The sigmoidal curve (■) represents the loss of enzymatic activity while the nebulizer is operating but where aerosol production is inhibited. The results are in stark contrast to the decline in activity observed by holding the LDH solutions in a water bath at 25 (◆) and 55°C (○). However, if the water bath is set at 60 °C (●) a rapid decline in activity occurs immediately. Data shown is the mean of n≧3 experiments.

sion and contraction of fluid elements and cavitational effects may induce changes in the protein conformation that reverse upon halting the process and are therefore not observed.

Influence of Additives on LDH Activity

The addition of 0.01%w/v Tween 80 does not dramatically influence the stability of the LDH during nebulization under normal conditions (Table 1). However, over the first few minutes of nebulization the loss of activity is less than that seen without the Tween. Similar results are seen with 1%w/v PEG 8000. Aerosolizing the LDH at 25°C in the presence of Tween 80 results in little loss of activity. The presence of PEG 8000 results in retention of all the activity for the duration of the experiment (Table 1). Thus, the Tween 80 and PEG 8000 appear to protect the LDH from inactivation resulting from aerosolization but not from heating. The PEG 8000 does provide some additional thermo-protection to the LDH during heating but the inactivation is only retarded with respect to time and not prevented.

Table 1. Influence of additives on enzymatic activity (a)

Nebulization time (min)	% of initial activity (100 %)					
	LDH alone		(+) Tween		(+) PEG	
	-	25°C	-	25°C	-	25°C
5	83±3	85±5	93±1	96±1	99±1	101±1
10	53±6	77±6	78±1	93±4	96±2	103±2
15	15±5	66±8	41±2	88±6	71±7	103±1
20	12±3	56±8	6±2	88±7	18±4	106±2

a Enzymatic activity of samples removed from the nebulizer reservoir.

Effects of Heating on Aerosol Solute Output

Although cooling of the nebulizer solution and the addition of a surfactant conferred protection to LDH it was noticeable that aerosol formation was reduced as the temperature dropped. We were therefore concerned that by solving one problem we were creating another. To quantify this effect, the solute (not weight) output was determined for a range of fixed operating temperatures between 10 and 50°C for a 10 minute period. As suspected, a large increase in output occurs as the temperature is raised (Fig 6). To examine this effect further the cumulative output was determined over 2 minute intervals for a total of 10 min. (a) The nebulizer was allowed to operate normally and (b) the temperature was fixed at the starting temperature of 21°C. Under normal operating conditions the output is non-linear and a patient inhaling aerosol will receive >5x more drug during the last 2 minutes of operation than during the initial 2 minutes (Table 2). This effect could be due to an increase in concentration of solute within the nebulizer, caused by evaporation of solvent. To check this, the solute concentration in the nebulizer solution was also monitored. Only a 5.5% increase in concentration occurred over 10 minutes of nebulization and this is not sufficient (21) to explain the large changes in solute output observed. Therefore, the increase must primarily be due to an increase in aerosol formation. In contrast, the output/min at 22°C is substantially reduced and approximately constant for the duration of the experiment (Table 2). These results have clinical implications and it will be important to remind patients that correct inhalation technique throughout the dosing period is crucial. Compliance and inhalation technique typically wanes during the latter stages of a dosing period and patients may therefore not receive an adequate amount of medication.

The reason for these observations may relate to the in-

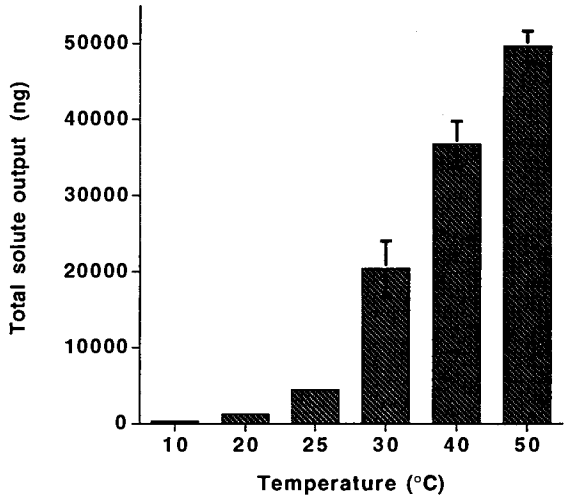

Fig 6 The recovered solute output of the nebulizer after 10 min operation at a range of fixed operating temperatures (10, 20, 25, 30, 40 and 50°C). The output was determined by spiking the LDH solutions with carboxyfluorescein (CF) to produce a 10μg/ml concentration of the fluorochrome and subsequently assaying the CF emerging from the nebulizer by spectrofluorimetry. A fixed flow of 5 Liters ambient air / min was allowed to flow through the nebulizer for these experiments. Data shown is the mean±standard deviation.

Table 2. Influence of temperature on cumulative solute output

Collection interval (min)	Heating (ng)	Heating (ng/min)	No heating (21 deg C) (ng)	No heating (21 deg C) (ng/min)
0 to 2	641	320	595	297
0 to 4	1961	490	1026	256
0 to 6	3418	570	791	132
0 to 8	6741	843	1318	165
0 to 10	9674	967	2450	245

fluence of temperature on viscosity. Viscosity changes will influence both cavitation and wave formation. Cavitation is influenced, among other factors, by the dynamic viscosity of the liquid. The capillary wave theory (14) predicts that capillary wave formation—and subsequent aerosol formation—will not occur until a threshold wave amplitude is induced by the ultrasonic oscillations. Once this threshold level is exceeded, the capillary waves will grow exponentially until instability at the wave crests produces droplets. The threshold amplitude of oscillation (A_t) required to generate capillary waves on the surface of the nebulizing fluid is given by

$$A_t = \frac{4*v}{f*\lambda_k} \quad (2)$$

where v is the kinematic viscosity (η/ρ), f is the frequency of oscillation and λ_k is the capillary wavelength (14). The viscosity of a pure Newtonian liquid will increase as temperature is decreased as

$$v = \frac{\eta}{\rho} = A_{vis}*e^{\left(\frac{E_{vis}}{RT}\right)} \quad (3)$$

where A_{vis} is a constant, and E_{vis} is the activation energy required to cause movement between two layers of liquid. It can be seen that A_t will be increased with decreasing temperature and a reduction in aerosol production can be expected. The enthusiasm for this argument is muted by the fact that the change in viscosity of pure water over the temperature range of interest 20–50°C is quite small ie ≈0.5 centipoise (25). It is not clear if this change is sufficient to explain the observed changes in output with temperature and further work is necessary to explore this issue.

Effect of Heat on Droplet Size

The mass median aerodynamic diameter (MMAD) of the droplets leaving the nebulizer at 20 and 50 degrees were 3.6±0.4 μm (n=6) and 3.9±0.2 μm (n=6) respectively. Thus, temperature has apparently little effect on the droplet size produced by the nebulizer. Heating caused a slight increase in the size distribution as shown in Fig 7 but it can be concluded that the loss of activity of LDH is not associated with any changes in the droplet size. The addition of 0.01%w/v Tween 80 also did not significantly alter the droplet size (data not shown).

SUMMARY

It has been shown that LDH is inactivated by the process of ultrasonic aerosolization. This effect can be reduced by preventing heating of the nebulizer solution during operation. By additionally including a surfactant such as Tween 80 to the protein solution almost all of the original activity can be retained. Unfortunately, cooling significantly reduces the solute output from the nebulizer rendering this approach impractical for the protection of thermolabile protein formulations. However, the results do serve to illustrate some features of ultrasonic nebulization that can destabilize protein solutions. Ultimately, the formulation requirements for ultrasonic nebulization of proteins may be quite different from those found suitable for air-jet nebulization and an important distinction must be made between the two modes of delivery in this respect.

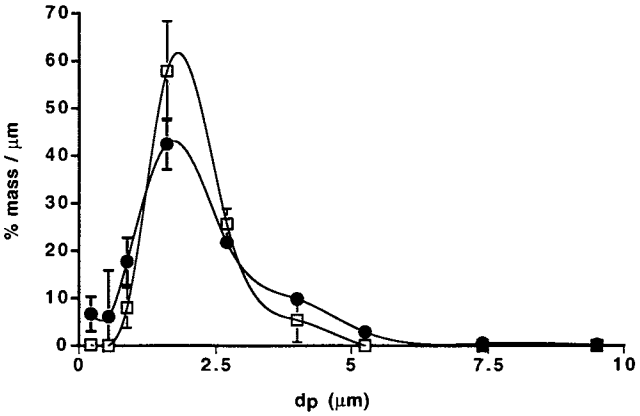

Fig 7 The influence of nebulizer solution temperature on the size of the generated aerosol. The size distribution of the dried solute (d_p) deposited in the Andersen cascade impactor is shown for the nebulizer solution held at 22 (□) and (●) 50°C.

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