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The Effect of Temperature on the Formation of Ethanol by *Candida Albicans* in Blood

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ABSTRACT: The effect of temperature on microbial fermentation in blood was studied. Specimens of human blood from a blood bank were inoculated with *Candida albicans*, an organism capable of causing fermentation. A preservative was added to a portion of the inoculated specimens. These inoculated specimens, as well as uninoculated blood, were stored under various temperature conditions. Production of ethyl alcohol was monitored over a period of six months. Fermentation was found to be highly temperature dependent, with refrigeration proving to be most effective at inhibiting ethanol formation.

KEYWORDS: forensic science, blood, *Candida albicans*, alcohol, temperature, ethanol

It has been shown that several microorganisms occasionally found in blood specimens are capable of producing ethyl alcohol [1,2]. Although Blume and Lakatua [1] found that sodium fluoride effectively inhibited alcohol production from a variety of microorganisms, one—*Candida albicans*—appeared to be unaffected by the addition of fluoride. *C. albicans* is commonly found in man, usually in the oral cavity and digestive tract, and less commonly in the vaginal tract of women. Though generally harmless, it can manifest itself as a pathogen. The organism has been called the most common and most serious pathogen of man [3]. The legal ramifications of this are obvious. If an organism common to man is capable of producing ethyl alcohol in stored blood, the question arises: Are the results of alcohol analysis reflective of an individual's level of intoxication or of post-sampling fermentation? With this in mind, we embarked upon a study of temperature versus ethanol production.

Method

Four plastic collection bags of human blood of 450-mL capacity were obtained from the Peninsula Memorial Blood Bank of Burlingame, California. Each bag contained dextrose (2.0 g), sodium citrate (1.66 g), citric acid (206 mg), monobasic sodium phosphate (140 mg), and adenine (17.3 mg). The blood was pooled and half of the pool was inoculated with *C. albicans* (Strain ATCC 14056). The inoculum was prepared to produce a final concentration of approximately 10 000 organisms per millilitre. This concentration was selected from a prior series of studies in which varying concentrations of this organism were cultured to assess optimum growth.

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The inoculated and uninoculated blood was divided among 112 10-mL Venoject® tubes. Of these tubes, 56 contained 100 mg of sodium fluoride and 20 mg of potassium oxalate. The other half contained no additives. The tubes were filled under nonsterile conditions with the uninoculated tubes serving as an experimental control over this study design. Representative sets of samples (A = uninoculated, -fluoride; B = inoculated, -fluoride; C = uninoculated, +fluoride; and D = inoculated, +fluoride) were then divided into temperature storage sets: refrigerated (6°C), room temperature (22°C), and body temperature (37°C). Specimens were analyzed after periods of 1 day, 2 days, 3 days, 5 days, 10 days, 35 days, and 6 months.

Analysis was performed by direct injection into a Hewlett-Packard 5880A gas chromatograph equipped with a flame ionization detector and a 6-ft by 1/8-in. (1.8-m by 3-mm) outside diameter (O.D.) stainless steel column packed with 0.2% Carbowax 1500 on 60/80 Carbopack C. Column temperature was 115°C. The injection port and detector temperatures were 180°C.

An 0.8-mL specimen of each sample was diluted with 3 mL of deionized water containing about 0.5% v/v of methyl ethyl ketone internal standard. Secondary alcohol standards of 0.220, 0.122, and 0.340% w/v ethyl alcohol were used to calibrate the instrument. Additionally, quality control samples (0.177% w/v ethyl alcohol) were analyzed at the beginning, end, and in the middle of each run. The minimum detectable concentration was determined to be 0.003% w/v ethyl alcohol.

Results

Duplicate analyses of the two pools, both inoculated and uninoculated, showed that no ethyl alcohol could be detected at time zero. Therefore, we could assume, with reasonable certainty, that any ethanol found during the period of study would be the product of microbial fermentation.

37°C

Two representative sets of bank blood were kept at body temperature and analyzed after periods of 28 h (one day) and 69 h (three days). Figure 1 shows that after 28 h at 37°C only two of the four specimens in Subset B (inoculated, -fluoride) produced ethyl alcohol in concentrations of 0.007 and 0.006% w/v. After approximately three days, Subset B showed

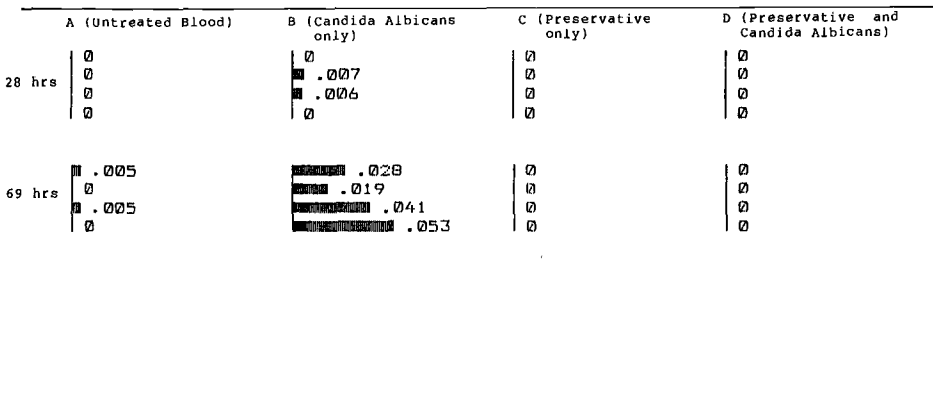


FIG. 1—Ethanol production in blood at 37°C after periods of 28 and 69 h. Values are in % w/v.

alcohol production in all four specimens: 0.028, 0.019, 0.041, and 0.053% w/v. Subset A, which had not been inoculated and contained no sodium fluoride, produced only a trace of alcohol after 69 h in two of four specimens. Both specimens contained 0.005% w/v ethyl alcohol. Specimens containing preservative, both inoculated and uninoculated, showed no detectable production of alcohol after three days at 37°C.

22°C

The room temperature sets of blood were analyzed after periods of 1, 2, 5, 10, 35, and 182 days. The results are illustrated in Fig. 2. As this figure indicates, the production of ethyl alcohol, once started after 5 days of incubation, was not affected by the presence of sodium fluoride. However, only the specimens inoculated with *Candida albicans* showed significant alcohol production. As with the 37°C sets of specimens, Subset A (uninoculated, — fluoride) showed slight (0.014 and 0.016% w/v) alcohol production in two of the four specimens. The uninoculated specimens that contained sodium fluoride (Subset C) showed no alcohol production even after 35 days of room temperature storage.

6°C

Five sets of specimens were kept under refrigeration and analyzed at 1, 5, 10, 35, and 182 days. No evidence of fermentation was found during the first 35 days. After 182 days, only a trace (0.004% w/v) of ethanol was found in 1 of the 4 uninoculated specimens that contained no sodium fluoride preservative. Of the 4 specimens inoculated with *C. albicans* that also contained no sodium fluoride, only 2 showed slight (0.008 and 0.015% w/v) alcohol production after 182 days of refrigerated storage. None of the preserved specimens, inoculated or uninoculated, showed any alcohol production after 182 days at 6°C.

	A (Untreated)	B (Candida only)	C (Preservative only)	D (Preservative and Candida)
1 day	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
2 days	0 0 0 0	.006 0 0 0	0 0 0 0	0 0 0 0
5 days	.016 .014 0 0	.039 .069 .16 .064	0 0 0 0	.071 .059 .065 .013
10 days	0 0 0 0	.048 .069 .04 .062	0 0 0 0	.07 .069 .037 .024
35 days (not analyzed)		(not analyzed)	0 0 0 0	.066 .083 .07 .038
182 days	.022 .007 .016 .021	.055 .074 .039 .066	(not analyzed)	(not analyzed)

FIG. 2—Ethanol production in blood at 22°C after periods of 1, 2, 5, 10, 35, and 182 days. Values are in % w/v.

Discussion

We studied the preservation and storage of blood specimens used for alcohol analysis, with emphasis on the issue of the loss or gain of ethanol over time. Winek and Paul [4] found no significant variation in alcohol content when blood specimens were analyzed within 14 days of collection regardless of the conditions of storage and preservatives present. This is in keeping with Glendening and Waugh's findings [5]. However, long-term storage has generally resulted in a loss of alcohol with time [5-7]. Also, it has been conceded that ethanol can be produced in blood specimens under certain conditions [1,2].

The issue of ethanol loss during long-term storage has been addressed in length in many publications. The preservation of blood with sodium fluoride has been shown to prevent effectively alcohol loss for up to two months when the specimens were stored at room temperature [5]. For longer periods of time, refrigeration was found to be necessary [5,8]. Meyer et al. [8] found the freezing of blood specimens to be most effective in preventing ethanol loss.

The issue of alcohol gain in blood specimens taken from living subjects has received less scrutiny. The instances of neof ormation of alcohol are less common. Nonetheless, the issue, both legal and scientific, remains.

Tests on postmortem blood specimens, which are more likely to exhibit neof ormation of ethanol, have shown that sodium fluoride is generally sufficient to preserve the integrity of the specimens [1,9]. However, Blume and Lakatua [1] found that sodium fluoride was ineffective in preventing ethanol production by *C. albicans*. Our study generally supports their conclusions. Although we detected no alcohol in a preserved group of specimens incubated at 37°C for 69 h, specimens that had been inoculated with *C. albicans* and stored at room temperature for more than five days showed significant alcohol formation. Furthermore, ethanol formation, once started in these inoculated specimens, generally increased although the absolute amount of ethanol formed appeared to be reaching a plateau concentration at approximately 0.08% w/v. On the basis that ethanol formation in blood would arise predominantly by the metabolic conversion of glucose, we calculated the maximum amount of ethanol that could be created by glucose fermentation. Using a blood glucose concentration of 95 mg/100 mL of blood, we calculated a first approximation value based upon the complete conversion of glucose to ethanol via the anaerobic glycolytic pathway in which 1 mole of glucose converts to 2 moles of ethanol. This calculated value is 0.05% w/v. The amount of ethanol formed in our study exceeded this value. We subsequently discovered that the blood from the Peninsula Memorial Blood Bank is treated not only with the addition of citrate but also 2.0 g of glucose per unit of blood. Therefore, our hypothesis of the maximum fermentation yield could not be assessed.

Room temperature storage of all specimens gave negligible or no ethanol formation until Day 5, and even under these conditions specimens that were uninoculated and contained fluoride formed no alcohol over a period of 35 days. Under refrigerated storage, none of the specimens showed any evidence of fermentation during the first 35 days, and only traces of alcohol were found after 6 months.

It appears that fermentation proceeds readily only by direct inoculation or contamination with *C. albicans*. Under such conditions the formation of ethanol is not inhibited by sodium fluoride. We have also found that the amount of alcohol formed over time is highly dependent upon the temperature of storage. Storage for approximately 1 day (28 h) at 37°C, 2 days at 22°C, and 35 days at 6°C produced no alcohol in specimens that were uninoculated and contained sodium fluoride as a preservative. Under these same storage temperatures and storage periods, the maximum amount of ethanol formation would be expected in inoculated and unpreserved specimens. Even in such specimens, the highest concentration of ethanol attained was 0.007% w/v.

Our study further showed that even when specimens were purposely inoculated with *C. albicans*, no alcohol formation took place for 69 h at 37°C if sodium fluoride at 10 mg/mL of blood was used as a preservative.

Therefore, it appears that legal questions regarding the issue of the neoformation of ethyl alcohol should be rendered moot if preservatives and short transport times are routinely used in bringing specimens to the laboratory and refrigeration is used in specimen storage.

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