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Postmortem Dating of Putrefied Material Through Ptomaine Estimation

Ptomaines, also known as putrefactive bases, are microbiological degradation products of postmortem protein catabolism and have caused much concern for forensic toxicologists for more than 100 years [*1,2*]. In 1885 Brieger [*3*] noted, in a review of the considerable volume of literature that existed even then, "that no facet of medical research, even extending into the present day, is so confused and devoid of actual results, as the field of the so-called putrefaction or cadaveric alkaloids." (The term ptomaine originates from Selmi [*4*] who, through the Stass-Otto method, succeeded in isolating from cadaveric material noncrystalline products that could be confused with morphine, codeine, and atropine in their reactions and effects.) Brieger's major contribution was to deviate from the standard practice of establishing such cadaveric alkaloids through experiments with aminals and chemical reactions by demonstrating the chemical individuality of the body and investigating its composition [*3*]. He succeeded in identifying (besides neurine, muscarine, dimethylamine, trimethylamine, and diethylamine) two diamines that he named "cadaverine" and "putrescine" that were then unknown to chemistry. Cadaverine was identified as pentylethyldiamine and putrescine was supposed to be a dimethylated ethyldiamine.

Brieger thought that the newly discovered products were bacterial decompositions of protein putrefaction, but he did not discover their formal genesis. This is understandable because the appropriate amino acids, lysine and ornithine, were discovered a few years later [*5*]. Through other studies Brieger's work was established and confirmed [*6–13*]. During the next 50 years his basic studies were greatly enhanced; certain well-defined proteins [*2,6–8,14*], pure cultures of microorganisms [*10,15–18*], and, ultimately, single amino acids [*12,19–21*] were used in putrefaction experiments.

As putrefaction products the ptomaines are the result of microbiological transpositions. Stodola [*22*] listed 15 such reactions, the most important being the decarboxylation of the amino acids to amines. This decarboxylation is a secondary step of postmortem protein catabolism [*23,24*] caused by bacterial decarboxylases [*25–28*] that were only partially identified until recently (Table 1). Degradation of the ptomaines (for example, of the amines by mono- and di-amineoxydases [*29–31*]) is also an effect of the cadaveric microflora.

Ptomaines remained within the domain of forensic toxicology until the present. Moreover, despite fundamental changes in analytical methods, there is still confusion regarding the toxicologically relevant substances, as evidenced in numerous publications throughout the last years, primarily from Italian [*32–35*], French [*36*], and Anglo-American authors [*37–41*]. As recently as 1969 Schmidt [*42*] stated that "the problems posed by the ptomaines are presently in no way solved; perhaps, in contrast to previous years, they are being neglected."

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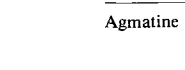
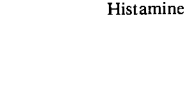
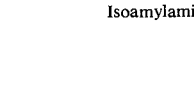
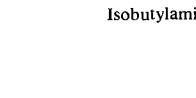
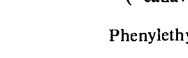
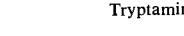
TABLE 1—*Occurrence in microorganisms of ptomaines and ptomaine-producing enzymes.*

Ptomaine	Formula	Enzyme	Enzyme Commission Number	Microorganism	Reference
Agmatine		arginine decarboxylase	4.1.1.11	*Clostridium propionicum*	66
β-alanine	$HOOC \cdot CH_2 \cdot CH_2 \cdot NH_2$	aspartate decarboxylase	4.1.1.19	*E. coli, B. subtilis*	26
γ-aminobutyric acid	$HOOC \cdot (CH_2)_3 \cdot NH_2$	glutamate decarboxylase	4.1.1.15	*E. coli, P. vulgaris, B. subtilis*	3, 15, 65
β-aminoisobutyric acid	$HOOC \cdot CH(CH_3) \cdot CH_2 \cdot NH_2$	unknown	...	...	...
Ethanolamine	$HO \cdot CH_2 \cdot CH_2 \cdot NH_2$	serine decarboxylase[a]	...	*E. coli*	65
Ethylamine	$CH_3 \cdot CH_2 \cdot NH_2$	alanine decarboxylase[a]	...	...	
Histamine		histidine decarboxylase	4.1.1.22	*E. coli, P. vulgaris*	16, 18, 65
Isoamylamine		leucin decarboxylase[a]	...	*E. coli, P. vulgaris*	68
Isobutylamine		valine decarboxylase[a]	4.1.1.14	*P. vulgaris*	66
Methylamine	$CH_3 \cdot NH_2$	glycine decarboxylase[a]	...	...	
β-methylbutylamine	$CH_3 \cdot CH_2 \cdot CH(CH_3) \cdot CH_2 \cdot NH_2$	isoleucin decarboxylase[a]	...	*P. vulgaris*	68
Pentamethylendiamine ("cadaverine")	$H_2N \cdot (CH_2)_5 \cdot NH_2$	lysine decarboxylase	4.1.1.18	*E. coli, B. subtilis, Bacterium cadaveris*	65, 66
Phenylethylamine		tyrosine decarboxylase[b]	4.1.1.25	*E. coli, Streptococcus faecalis*	68
Tetramethylendiamine ("Putrescine")	$H_2N \cdot (CH_2)_4 \cdot NH_2$	ornithine decarboxylase	4.1.1.17	*E. coli, P. vulgaris, B. subtilis*	65, 66
Tryptamine		tryptophan decarboxylase	4.1.1.27	*P. vulgaris*[c]	67
Tyramine		tyrosine decarboxylase	4.1.1.25	*E. coli, S. faecalis*	68

[a] Existence not proven.
[b] Decarboxylates and phenylalanine.
[c] Controversial.

For the thanatologist it is interesting that the appearance of ptomaines in cadaveric material seems to be time-related. For example, Brieger [*3*] noted that although traces of cadaverine and putrescine could sometimes be identified after 3 or 4 days, a quantity sufficient for analysis could be detected only after 11 to 14 days postmortem.

Time-related aspects of the development of ptomaines were not mentioned again in the literature until the second part of this century, when Berg and Laves [*43,44*] noted an early, gradual decrease in histamine concentration in the blood that lasted approximately 40 h. When postmortem histamine concentrations were elevated, differentiation had to be made between an agonal concentration increment and a postmortem synthesis. The latter possibility was presumed when a tyramine increment, which occurs exclusively postmortem, could be demonstrated (at a maximum concentration of 10 mg/litre). Watanabe [*45*] discovered an increase in amine concentrations between 10 and 20 days postmortem. Exact identification and quantitation were not possible. Italian authors [*33–35*], who also did not succeed in establishing an exact identification, noted an increase in activity of the putrefactive bases in parenchymatous organs between the third and fourth day postmortem. No reference to temperature was made. Schmidt et al [*46,47*] observed a consistent increase in the concentration of amino groups in putrefying liver homogenate after approximately 10 days. As the carboxyl group concentration did not rise accordingly, the amine production was considered responsible for this effect. In material stored at 4°C, Kaempe [*48–51*] observed tyramine concentrations greater than 3 mg/litre from the start of the second week postmortem and greater than 10 mg/litre after the third week as well as phenylethylamine at corresponding times and concentrations. The highest registered concentrations were 200 mg/litre. Browers [*52*] noted β-alanine after eight days in striated muscle stored at 2°C, a finding which Cantoni et al [*53*] could not reproduce.

Experimental Procedure

Materials

Our investigations were carried out on stored blood, liver homogenate, gallbladder bile including extravesicular putrefactive transudate, and putrefactive thoracic fluid from animal cadavers.

Blood was obtained from the carotid artery of recently slaughtered cattle and collected in seven jars sealed with a perforated lid and sterile cotton pads. One of six pure cultures of common putrefaction microorganisms (*Bacillus subtilis, Candida albicans, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Serratia marcescens*) was injected into each of six samples; the seventh sample remained untreated.

Human liver was homogenated by an Ultra-Turrax apparatus and divided into seven portions. Single portions were poured into seven sterile plastic containers and sealed as above. Four samples were stored until putrefaction occurred, two at room temperature (20°C), one at 35°C, and one at 3°C. The other samples were kept at 20°C; the fifth sample was continuously treated with oxygen, and the sixth, with nitrogen. One percent gentamicin was applied to the seventh homogenate.

The gallbladders were removed from recently slaughtered pigs and were carefully dissected from the surrounding liver tissue. Each was placed in a large plastic chamber fitted with a tightly screwed cap. A plastic plate had been previously arranged at a slight slant so that an appropriate floor with an underlying hollow receptacle was formed. One hose was inserted into the hollow space and another into the gallbladder. Through this arrangement it was possible to aspirate the internally and externally accumulated bile separately. Duplicate samples were taken at regular intervals.

Healthy dogs of different breeds were killed through a narcotic injection and the cadavers were placed in a large enamel basin which was then carefully sealed. Deposition of dipteran eggs and maggot grubs was prevented by regular treatment with an insecticide spray. The experiments were carried out at a fairly constant temperature of 10°C. Thoracic fluid was aspirated from the fifth day on.

Procedure

From all test preparations samples were taken at regular intervals and stored in a deep freeze for collective examination. Protein was precipitated from the liquid medium by adding 2 ml of 10% trichloroacetic acid to 1 ml of substrate. The use of 96% ethanol (3 ml to 1 ml of substrate) gave the best results in the workup of the liver homogenate. The other methods were as described by Bonte and Rustemeyer [*54*]. The separation of the protein catabolites was carried out with two-dimensional thin-layer chromatography, and the identification was performed with the ninhydrin-reaction test by comparison with commercially marketed amines and their related substances. Quantitative analysis was accomplished with a densitometer.

In addition, all samples were treated in a second, completely different manner by preparation of the dinitrophenol (DNP) derivatives as recommended by Tancredi and Curtius [*55*]. The separation of the DNP-amines was carried out on silica-gel thin-layer plates. In the first dimension chloroform:methanol:glacial acid (100:2:1) was used, and in the second, benzol:ethyl acetate (1:1). The identification of the intense yellow spots was possible through comparison with previously prepared reference substances.

Results

The results of the putrefaction experiments with untreated blood are shown in Fig. 1. A total of eleven ptomaines could be demonstrated, of which β-aminoisobutyric acid, histamine, putrescine, and tyramine were present only in traces or in the later trial phase and β-alanine and γ-aminobutyric acid appeared from the very beginning.

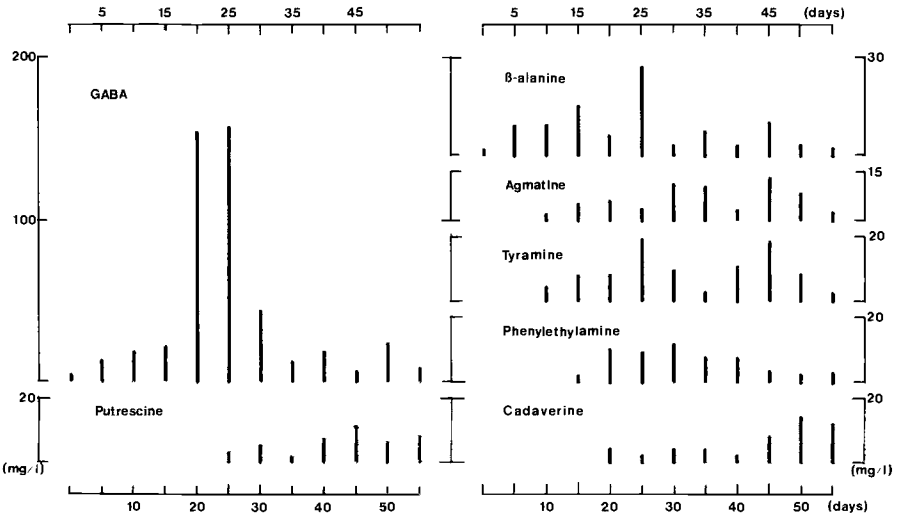


FIG. 1—*Ptomaines in stored blood; in-vitro concentration changes at 20°C. Note the change in scale for* GABA (γ-*aminobutyric acid). Ordinate: concentration in mg/litre blood; abscissa: storage time in days.*

Very interesting alterations were found after the inoculation of putrefactive microorganisms. In the experiment with *Bacillus subtilis* (Fig. 2) the ptomaines appeared later, as in untreated blood; agmatine, γ-aminobutyric acid, cadaverine, and putrescine reached evidently higher concentrations. Bacteriological controls showed a distinct multiplication of the inoculated species but no superinfection.

In the study with *Proteus vulgaris* (Fig. 3) a biphasic increment of γ-aminobutyric acid,

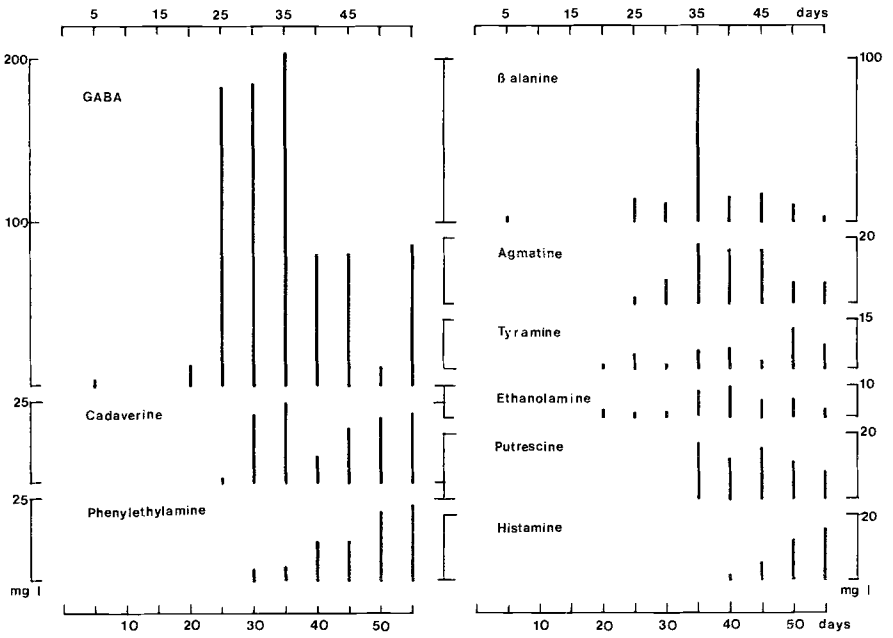


FIG. 2—*Ptomaines in stored blood; in-vitro concentration changes after inoculation of* Bacillus subtilis *at a storage temperature of 20°C. Note the change in scale for* GABA (*γ-aminobutyric acid*) *and β-alanine. Ordinate: concentration in mg/litre blood; abscissa: storage time in days.*

histamine, and putrescine was noticed. This alteration became conspicuous with γ-aminobutyric acid in particular. Ethanolamine could be demonstrated in a higher concentration than in untreated blood, whereas β-alanine remained at a negligible level.

Pseudomonas aeruginosa caused only poor amine production. The ptomaines could be identified only in traces and at very late trial phases. After inoculation of *Candida albicans* no significant changes developed as compared to untreated blood. In contrast, the experiment with *Serratia marcescens* induced a certain acceleration and intensification of the production of agmatine, γ-aminobutyric acid, cadaverine, and histamine (Fig. 4). Only β-alanine remained at a low concentration. As in all cases no superinfection could be demonstrated.

The most striking alterations could be seen after treatment with *Escherichia coli* (Fig. 5). The ptomaines agmatine, ethanolamine, γ-aminobutyric acid, cadaverine, histamine, and putrescine appeared during the initial phase and then decreased more or less evenly. Between 30 and 35 days postmortem another reduction occurred with nearly all ptomaines.

The results of our experiments with liver homogenate at 20°C resembled those of untreated blood (Fig. 6). Also, β-alanine and γ-aminobutyric acid could be shown from

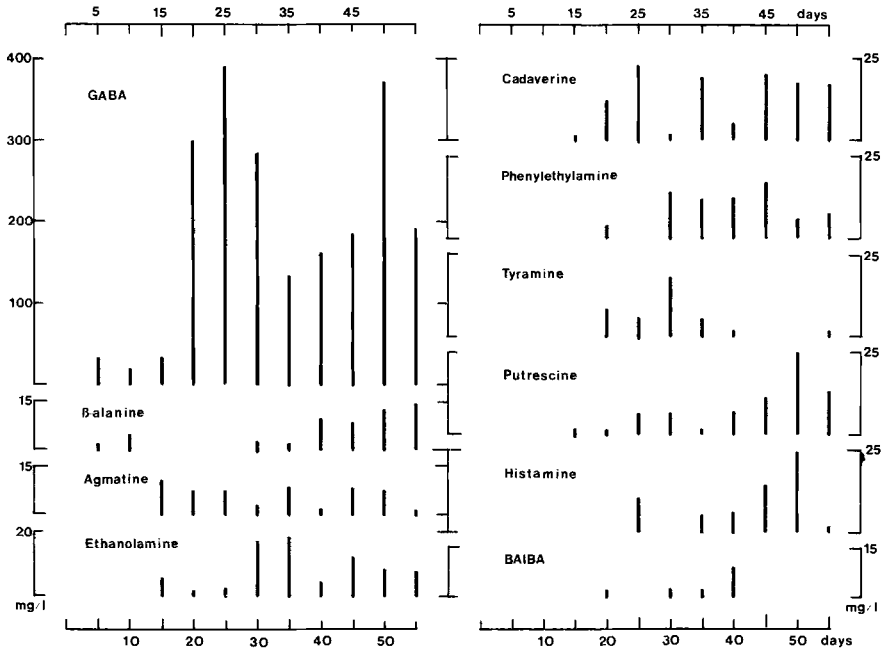


FIG. 3—*Ptomaines in stored blood; in-vitro concentration changes after inoculation of* Proteus vulgaris *at a storage temperature of 20°C. Note the change in scale for* GABA (γ*-aminobutyric acid). Ordinate: concentration in mg/litre blood; abscissa: storage time in days.* BAIBA = β*-aminoisobutyric acid.*

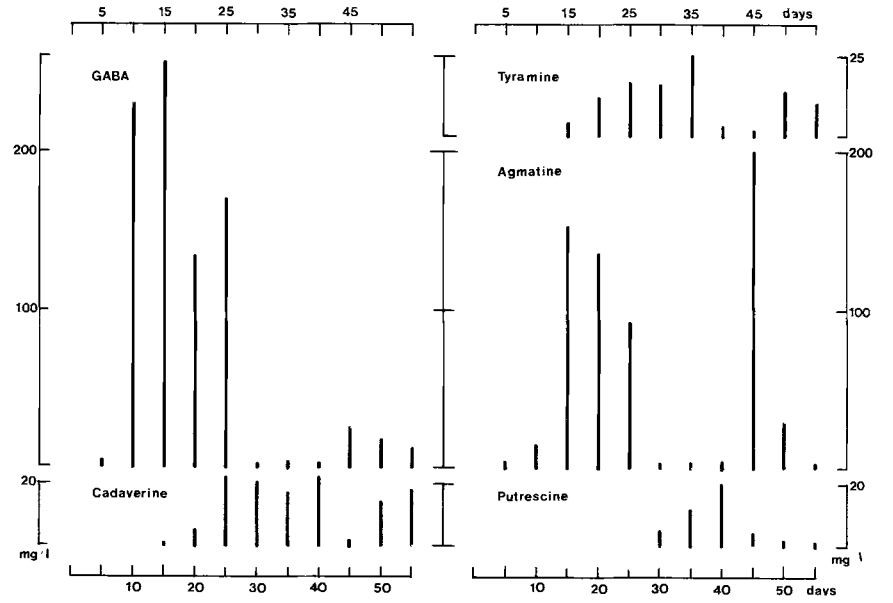


FIG. 4—*Ptomaines in stored blood; in-vitro concentration changes after inoculation of* Serratia marcescens *at a storage temperature of 20°C. Note the change in scale for* GABA (γ*-aminobutyric acid) and agmatine. Ordinate: concentration in mg/litre blood; abscissa: storage time in days.*

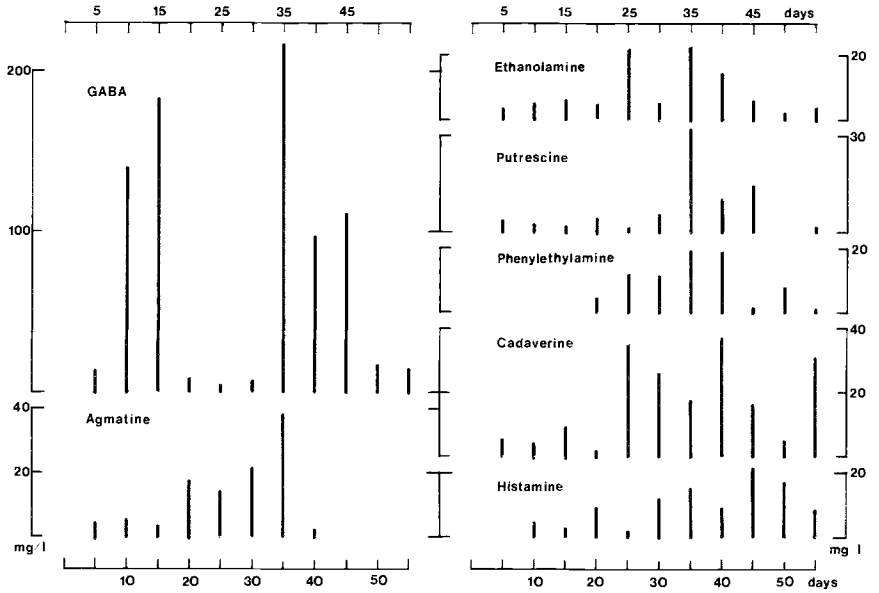


FIG. 5—*Ptomaines in stored blood; in-vitro concentration changes after inoculation of* Escherichia coli *at a storage temperature of 20°C. Note the change in scale for* GABA (γ-*aminobutyric acid). Ordinate: concentration in mg/litre blood; abscissa: storage time in days.*

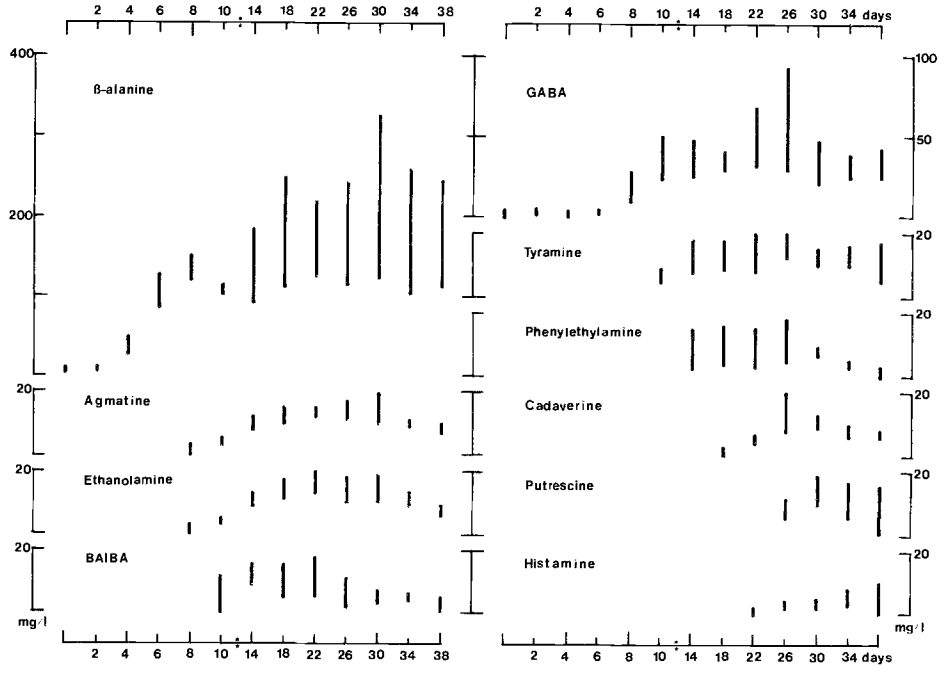


FIG. 6—*Ptomaines in liver homogenate; in-vitro concentration changes at 20°C shown as the ranges of two single estimations. Note the change in scale for* GABA (γ-*aminobutyric acid) and* β-*alanine and the break in the abscissa between 10 and 14 days marked by asterisks. Ordinate: concentration in mg/kg liver; abscissa: storage time in days.* BAIBA = β-*aminoisobutyric acid.*

the beginning, whereas cadaverine, histamine, putrescine, and tryptamine were characteristic of the later putrefaction phase. Phosphoethanolamine was detected in some samples about the third week postmortem.

The liver experiments showed a considerable deviation in results at temperatures of 3 and 35°C (Figs. 7*a* and *b*). In both trials fewer ptomaines emerged than at 20°C and, with one exception, were present in much lower concentrations. The one exception was

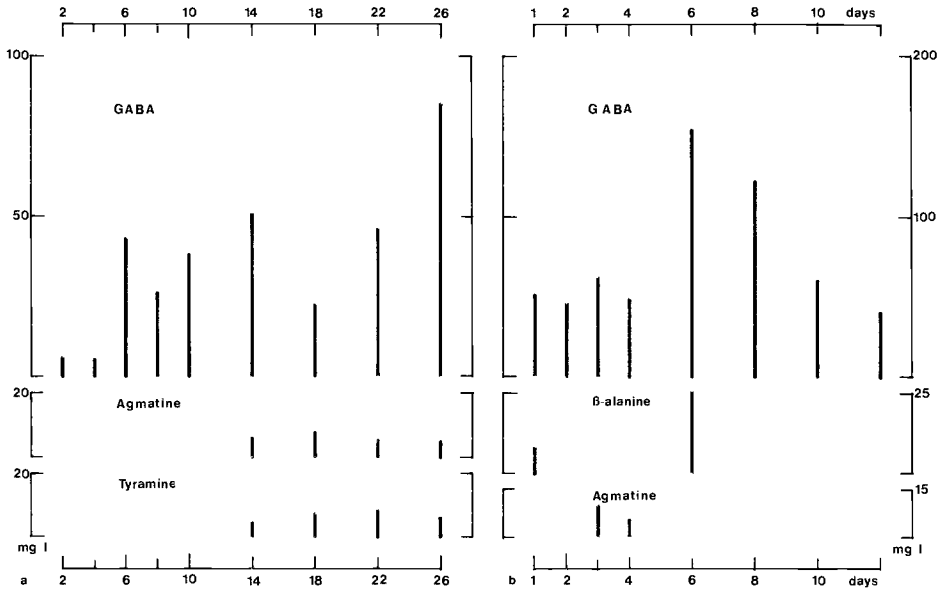


FIG. 7—*Ptomaines in liver homogenate;* (a) *in-vitro concentration changes at 3°C and* (b) *in-vitro concentration changes at 35°C. Note the change in scale for* GABA (γ*-aminobutyric acid) and the great differences in scales between* (a) *and* (b). *Ordinate: concentration in mg/kg liver; abscissa: storage time in days.*

γ-aminobutyric acid, which did not behave differently at lower temperatures than at room temperature. At higher temperatures it appeared more rapidly and reached high concentrations, only to then vanish quickly.

In the oxygen perfusion trials at 20°C it could be noted that the few identified putrefaction products (agmatine, β-alanine, and γ-aminobutyric acid) that appeared during the entire experiment were present only sporadically and even then in trace amounts. In the nitrogen perfusion trials (Fig. 8) a considerable concentration increment was evident. The maximum values, however, stayed below those of the untreated liver homogenate. Similarly, more rapid appearance of the ptomaines correlated with quicker fall in concentration. No ptomaines could be found after the addition of an antibiotic.

In the gallbladder bile only six decarboxylation products could be identified (Fig. 9), of which only β-alanine and γ-aminobutyric acid appeared regularly, whereas β-aminoisobutyric acid, agmatine, phenylethylamine, and tyramine could be demonstrated only sporadically and at later trial times. No apparent differences could be found between gallbladder bile and extravesicular putrefactive transudate (Fig. 10).

In the putrefactive fluid from the thoracic cavity of dog cadavers a total of nine ptomaines were documented (Fig. 11), of which β-aminoisobutyric acid, phenylethylamine, and putrescine occurred only in traces and at later periods and β-alanine and γ-amino-

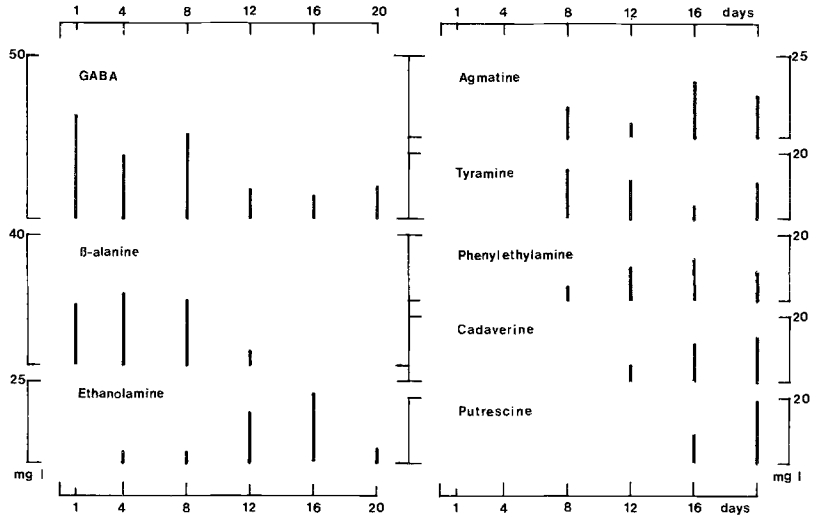


FIG. 8—*Ptomaines in liver homogenate; in-vitro concentration changes during nitrogen perfusion at 20°C. Ordinate: concentration in mg/kg liver; abscissa: storage time in days.*

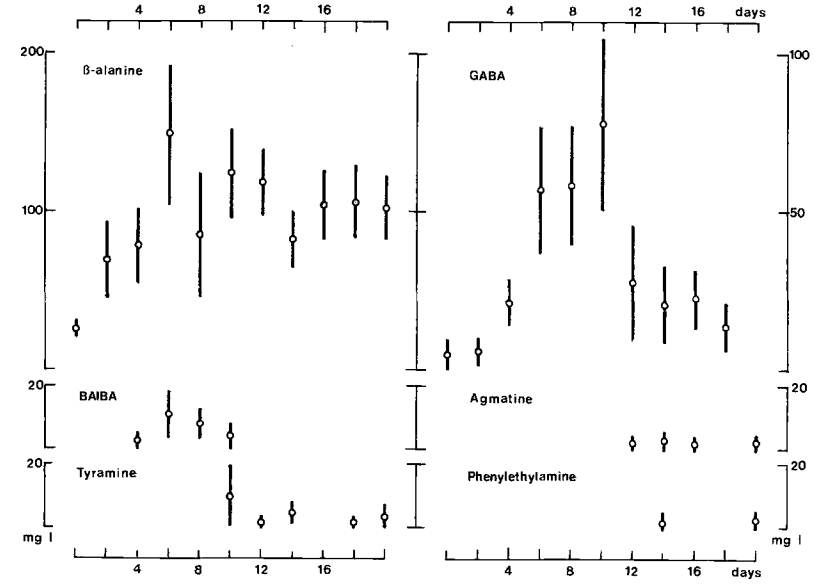


FIG. 9—*Ptomaines in gallbladder bile; postmortem intravesicular concentration changes at 20°C shown as the means of five single estimations and confidence limits. Note the change in scale for β-alanine. Ordinate: concentration in mg/litre bile; abscissa: postmortem time in days.* BAIBA = *β-aminoisobutyric acid.*

butyric acid could be identified from the beginning of the trial. Tyramine appeared from 10 days on, agmatine from 15, and ethanolamine and cadaverine from 20 days postmortem.

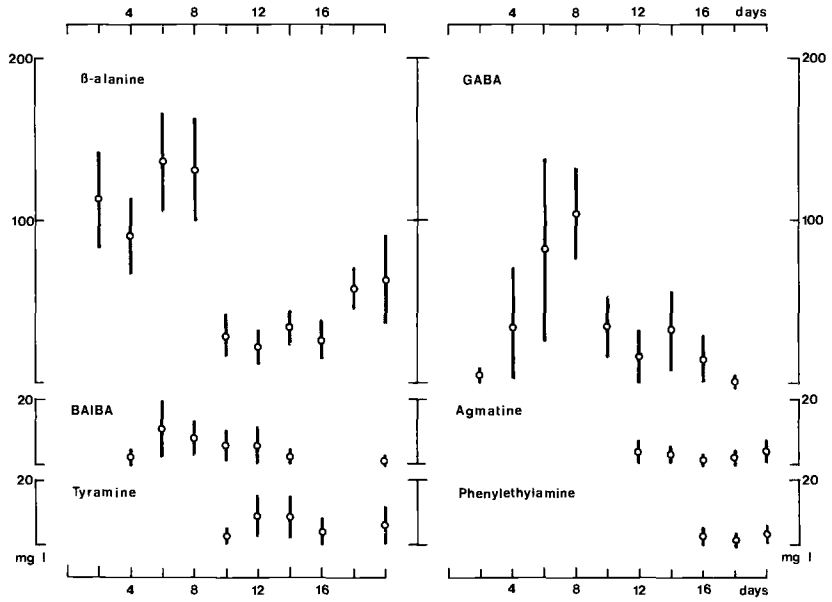


FIG. 10—*Ptomaines in gallbladder bile; postmortem concentration changes in extravesicular transudate at 20°C trial temperature shown as means of five single estimations and confidence limits. Note the change in scale for* GABA (γ*-aminobutyric acid) and* β*-alanine. Ordinate: concentration in mg/litre transudate; abscissa: postmortem time in days.* BAIBA = β*-aminoisobutyric acid.*

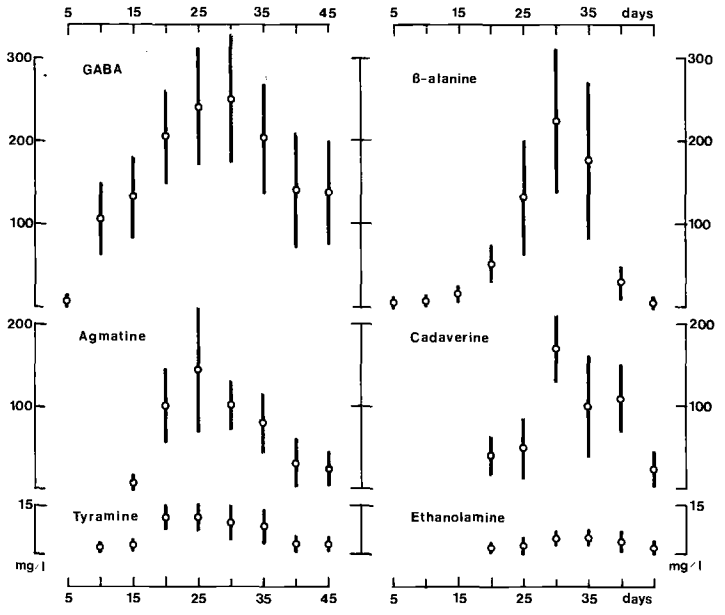


FIG. 11—*Ptomaines in putrefying transudate from the thoracic cavity of the dog; postmortem in-situ concentration changes at 10°C shown as means of five single estimations and confidence limits. Note the change in scale for tyramine and ethanolamine. Ordinate: concentration in mg/litre transudate; abscissa: postmortem time in days.*

Discussion

From those amines mentioned in the literature which were identified in our putrefaction materials, three (β-aminoisobutyric acid, ethanolamine, and phosphoethanolamine) emerged in hardly more than trace amounts. The five alkyl-amines could not be detected at all with the methods used in the experiments and also could not be demonstrated in comparative tests with the appropriate pure substances. Only limited data are available in the literature on the maxima for amine concentrations. The classical putrefaction diamines, cadaverine and putrescine, which never appeared in concentrations higher than 20 mg/litre in our experiments, were never quantitated by previous researchers. Brieger [*3*] mentioned that the preparation of 10 to 20 kg of raw material was necessary to gain analytically sufficient quantities. Berg [*43*] reported a postmortem neogenesis of histamine and tyramine reaching a magnitude of approximately 10 mg/litre. Kaempe [*48,50*] also found tyramine in stored organs in concentrations up to 10 mg/litre and, on occasion, up to 200 mg/litre after several years of storage. Because Kaempe experimented at 4°C, a relatively significant correlation could be obtained with our observed tyramine concentrations, which were around 50 mg/litre. Further, Kaempe [*50*] demonstrated phenylethylamine in concentrations of approximately 3 to 9 mg/litre, which correlates well with our results.

Very important is the time relation of the appearance of the individual ptomaines during the putrefaction process, which indicates a staggered arrangement. At the beginning of our experiment β-alanine and γ-aminobutyric acid were already present in all materials under investigation, but in low concentrations. Then the ptomaines agmatine, ethanolamine, and tyramine appeared. Even later a third group containing cadaverine and phenylethylamine evolved. Lastly, histamine and putrescine emerged.

Corresponding observations concerning the first and the last group of putrefactive amines are lacking in literature. The time interval for our second group to appear was 8 to 10 days. Comparative specifications were submitted only by Kaempe [*48*], who reported tyramine to be present at 4°C at a concentration of 10 mg/litre from the third week on, favorably concurring with our data. Furthermore, a correspondence in time exists between the data of Watanabe [*45*] and Schmidt et al [*47*], where amines occurred after the tenth day at the earliest; however, the amines were not further identified.

Our cadaverine-phenylethylamine group was first identified at 20°C approximately between 14 and 20 days, and at 10°C between 20 and 25 days. Brieger [*3*] detected traces of cadaverine after three days that with time continuously increased in concentration. Because no information concerning the absolute quantities were provided and large amounts of putrefying material was processed, it seems entirely feasible that the margin of detection of the results presented here (about 1 mg/litre) was reached by Brieger at later times. Marozzi and Lodi [*34*] also observed cadaverine formation around the third to fourth day postmortem. Because the experiments were interrupted after the fourth day, specifications regarding temperature were lacking, and definite identifications were not carried out, conclusive comparison seems hardly possible. Kaempe [*50*] detected phenylethylamine between 23 and 49 days postmortem, which conforms with our own observations.

When the experimental temperature was changed, an expected reduction was observed at 3°C and an increase at 35°C of ptomaine production in liver homogenate could be noted. However, in both trials a considerable inhibition of most decarboxylases was observed which was congruent to data of other investigators, indicating that the optimal temperature for the microbic decarboxylases was about 20°C [*47,56*]. Only the production of γ-aminobutyric acid appears to be increased rather than suppressed at a temperature of 35°C. It is controversial whether production of the ptomaines occurs chiefly in an aerobic or an anaerobic milieu. Although most previous investigators described de-

carboxylation processes accelerated during oxygen perfusion and decelerated in a nitrogen atmosphere [*3,56,57*], others assumed an inverse effect [*19,47*]. In our own experiments it could be shown that under pure oxygen perfusion ptomaine formation commenced at a considerably retarded rate and reached conspicuously low concentrations. With nitrogen, on the other hand, a considerable acceleration in the decarboxylation processes began although higher maximal values were not reached. With all amines the concentrations receded rather rapidly.

After death the further breakdown of the proteogenous amino acids is accomplished by foreign enzymes because the auto-enzymes, after complete exhaustion of the energy-spending systems, are blocked. Thus the microflora plays an important role in the elimination of protein catabolites. In our experiments with some ubiquitous species belonging to the common putrefaction flora [*58–63*], it could be demonstrated that the different species, depending on their enzyme content, were involved in this secondary step of postmortem protein degradation to a variable extent. That *Bacillus subtilis, Escherichia coli,* and *Proteus vulgaris* are provided with decarboxylases has already been mentioned by previous authors [*64–68*]. *Serratia marcescens* and *Candida albicans* also seem to have these enzymes. Only *Pseudomonas aeruginosa* did not demonstrate such activity; however, the species is believed to contain transaminases [*69*] and deaminases [*26,70*].

Because amino-acid degradation is effectuated exclusively by microbial enzymes, amine production should be suppressed after disposal of the microflora. As expected, this effect did occur when antibiotics were added to the putrefaction trial with liver homogenate. No amines could be identified during the entire test series. Despite the apparent differences in the amine production of the specific bacteria it is very interesting that similar concentration changes occurred in all natural putrefactive substrates under investigation. It seems that more or less stable catabolic conditions arise with the usually developing mixed flora, probably because of net effects between the individual bacterial performances. There are some exceptions, such as an impending infection or previously initiated antibiotic therapy [*71,72*].

Summary

Putrefaction experiments were carried out on blood, liver homogenate, gallbladder bile including extravesicular transudate, and putrefactive fluid from the thoracic cavity of dog cadavers. The ptomaines (putrefactive amines) were identified with two-dimensional thin-layer chromatography. The changes in concentration were estimated through densitometry and related to postmortem intervals. The investigations had the following results.

1. Up to twelve ptomaines could be identified. The postmortem alterations were similar in all substrates under investigation and were characterized by a time-dependent gradation. During the first week, β-alanine and γ-aminobutyric acid were observed. At a storage temperature of 20 °C, agmatine, ethanolamine, and tyramine appeared during the second week, cadaverine and phenylethylamine during the third, and putrescine and histamine during the fourth week or later.

2. Experiments after inoculation of some ubiquitous microogranisms of common putrefaction flora showed very specific alterations, indicating that the respective *Bacillus, Candida, Escherichia, Proteus,* and *Serratia* species are provided with amino-acid decarboxylases and thus are involved in postmortem amino-acid catabolism. Amine production ceased after the addition of antibiotics.

3. Putrefaction at 35 °C caused a certain acceleration, whereas studies at 3 °C produced an inhibition, of ptomaine production. With the exception of γ-aminobutyric acid, in both studies fewer amines appeared and were present in lower concentrations that at 20 °C. Since experiments with dog cadavers at 10 °C were characterized by a deceleration

in the appearance of the amines, the results imply that the optimal temperatures for the microbial amino-acid decarboxylases are around 10 to 20°C.

4. Oxygen perfusion induced a nearly complete cessation of ptomaine production, whereas the anaerobic nitrogen atmosphere results in an increased and accelerated amine production.

5. Ptomaine analysis of cadaver material can be important in estimating the age of a corpse. The appearance of the individual ptomaines at various times allows discrimination by at least weekly postmortem intervals, even when the analysis is restricted to qualitative identification. It is possible that when quantitative conditions are considered a more precise classification could be obtained.

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