aluminum in serum, there is no general agreement regarding optimal control of matrix effects with this technique (5). Several studies performed with the stabilized temperature platform have strongly recommended the utilization of magnesium nitrate diluent (2, 3). The studies we report were prompted by our inability to completely eliminate the matrix effects of serum with this diluent (see above). Others using a magnesium nitrate diluent have also reported matrix effects with serum and have recommended standardization with protein-containing solutions (2) or a protein precipitation technique (6). The basis for our new procedure is the finding that these matrix effects can be eliminated with a diluent of ammonium hydroxide/sulfuric acid. The fact that the new procedure can be standardized with aqueous standards makes this methodology distinctly advantageous.

The accuracy and precision of the procedure and the values observed in normal individuals are similar to results obtained by us previously (1) and by others (2, 3).

Nonetheless, the proposed methodology has several disadvantages worth noting. The heating cycle is relatively long, which slows the rate at which samples can be run. Thus far, our efforts to significantly decrease the duration of the heating cycle have been unsuccessful. The need to use prediluted samples is another disadvantage. It would obviously be much more convenient to assay serum samples directly, but our attempts to use undiluted serum have not been successful, presumably because the matrix modifier does not mix easily with undiluted serum.

Finally, we recognize that our method yields only a small absorbance value for serum containing physiological (<10 ng/L) amounts of aluminum. In this instance we have found that scrupulous care must be taken to avoid contamination because contamination is the major limiting factor. In addition, we recommend the routine use of triplicate dilutions in this concentration range to improve the absolute precision of the assay.

References

Table 2. Analytical Recovery Data

<table>
<thead>
<tr>
<th>Sample</th>
<th>Range in sample</th>
<th>Added</th>
<th>Mean (SD) recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal serum</td>
<td>0–6</td>
<td>95</td>
<td>96 (3.6)</td>
</tr>
<tr>
<td>Hemodialysis serum</td>
<td>18–61</td>
<td>83</td>
<td>85 (2.6)</td>
</tr>
<tr>
<td>Normal urine</td>
<td>4.5–10</td>
<td>86</td>
<td>87 (2.4)</td>
</tr>
<tr>
<td>n = 5 each.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Precision Data for Serum Samples

<table>
<thead>
<tr>
<th>N</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within run</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>4–6</td>
<td>5</td>
<td>0.6</td>
<td>12</td>
</tr>
<tr>
<td>10</td>
<td>95–109</td>
<td>102</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Between run</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15*</td>
<td>43–57</td>
<td>50</td>
<td>4</td>
<td>8</td>
</tr>
</tbody>
</table>

* Measured over a 10-week period.

Influence of Blood Pressure, Heart Rate, Age, and Sex on Concentrations of Atrial Natriuretic Factor and Cyclic GMP in 124 Volunteers

Marion Wencker, Susanne Hauptlorenz, Willi Moll, and Bernd Puschendorf

The significance of increased atrial natriuretic factor (ANF) in relation to blood pressure and age is still controversial. We investigated the influence of blood pressure, age, and some other variables on ANF and its putative second messenger, cGMP. Samples for ANF and cGMP detection were taken from 124 ostensibly healthy individuals who were donating blood. Samples were also collected from 27 volunteers before and after blood donation, to study the influence of bleeding. During blood donation, ANF increased from 78.9 to 87.4 ng/L (P = 0.0035), whereas cGMP remained unchanged. ANF concentrations in 124 healthy individuals, corrected for the influence of bleeding, were 61.5 (SD 26.1) ng/L, with a 95% confidence interval of 10.0 to 112.1 ng/L. Mean cGMP concentrations in plasma were 2.9 (SD 1.45) mmol/L, with a 95% confidence interval of 0.4 to 5.75 mmol/L. Multivariate analysis revealed no significant influence of blood pressure, age, heart rate, or sex on concentrations of either ANF or cGMP in plasma.

Additional Keyphrases: variation, source of · reference values

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We would like to dedicate this paper to Univ.-Prof. Dr. Franz Dienstl on his 60th birthday.

Received February 3, 1989; accepted April 11, 1989.
Since deBold et al. (1) first described natriuretic activity of rat atria extracts, much research concerning physiological and pathophysiological action of the atrial natriuretic factor (ANF) has been done. It is well known that patients with congestive heart failure (2, 3) and arrhythmias (4, 5) such as atrial fibrillation and atrial flutter have significantly increased ANF concentrations.

Recent data (6–8) suggest that cyclic GMP (cGMP) is the "second messenger" of ANF. Like ANF, it is increased in patients with various heart diseases (9–11). A significant correlation of cGMP with right atrial pressure and other hemodynamic variables, as well as with clinically assessed severity of congestive heart failure (10), has been shown. Owing to its high in vitro stability and easy pre-analytical handling, cGMP may in the future become a routinely measured analyte in cardiology. As far as we know, no studies have focused on age- and blood pressure-dependent changes in cGMP.

Profound knowledge of the physiological states of a system and of its modifying factors is needed to define pathological conditions. With the determination of normal values, the sensitivity and specificity of various measurements can be assessed. They are useful tools for evaluating both the quality of the methods and the epidemiological implications of the results.

However, contradictory results have been reported concerning the influence of blood pressure on ANF concentrations in plasma. Some (12–16) have found increased ANF in patients with hypertension and describe a positive correlation of ANF with blood pressure; others (17, 18) could find no influence of blood pressure on ANF. Some investigators report an increase of ANF in the elderly (19–21), but only a few patients were studied in most reports, and patients with heart diseases have not always been excluded (19).

The aim of this study was therefore to investigate

(a) ANF and cGMP concentrations in plasma and their correlation with blood pressure in nonhypertensive, nonhospitalized individuals;
(b) dependence of ANF and cGMP on heart rate, age, and sex;
(c) the influence of blood donation on ANF, cGMP, and other variables; and
(d) establishment of reference values for a representative number of healthy individuals for the analytical methods described below.

Blood donors are a valuable group of healthy individuals who seem suitable as sources of normal values in a representative number of persons. We investigated ANF and cGMP in such subjects in relation to blood pressure, heart rate, age, sex, and factors of physical constitution.

Materials and Methods

For ANF and cGMP determination, plasma was separated by centrifugation immediately after blood withdrawal, frozen in liquid nitrogen, and stored at -20 °C until assayed. The stability of ANF in plasma at room temperature is critical, but immediate processing yielded recovery rates of 97.9% (22). No significant deterioration was observed on prolonged storage at -25 °C (22). ANF concentrations in plasma were measured by means of a commercially available 125I-labeled radioimmunoassay without prior extraction (from Eiken, Tokyo, Japan; purchased from Behring-Institut, Vienna, Austria). Samples were assayed in one run; the intra-assay coefficient of variation (CV) was 11.2%.

For cGMP determination we used a 3H-labeled radioimmunoassay (from Amersham International Ltd., Amersham, Bucks., U.K.) after extraction of samples with ether. cGMP in EDTA plasma was stable at room temperature and at 4 °C for several days (unpublished observations). The intra-assay CV was 6.4%; the interassay CV was 13.6%.

Protocol I. Ethical and technical considerations make it desirable to submit donors to only one venipuncture. For this reason, and to prevent contamination, sample collection in routine blood donation must take place at the end of the donation period. To correct the data for the influence of blood donation on ANF and cGMP, we followed an extended protocol in a small number of volunteers. Twenty-seven such volunteers gave informed consent. Before blood donation they were carefully questioned by a specially trained physician for a history of hypertension, cardiac or thyroid disease, as well as medical therapy. Age, height, weight, and heart rate were recorded. Blood pressure was measured before and after blood donation. For the determination of ANF and cGMP, as well as standard erythrocyte measurements, we obtained one sample before and one after the donation of 450 mL of blood. Blood specimens for erythrocyte count and mean corpuscular volume were analyzed with a standard cell counter (Coulter Electronics, Hialeah, FL). Hemoglobin was determined by the cyanhemoglobin method. The hematocrit value was calculated as erythrocyte count multiplied by mean corpuscular volume. The quotient of hemoglobin and erythrocyte count gave the mean corpuscular hemoglobin. The mean corpuscular hemoglobin concentration was calculated as hemoglobin divided by erythrocyte count multiplied by mean corpuscular volume. In these cases we used special blood bottles that allowed separate blood withdrawal without contamination directly after venipuncture.

Protocol II. For determination of normal ANF and cGMP values, we investigated 139 healthy blood donors at two blood donation rallies of the Austrian Red Cross. Ten individuals who were receiving constant medication were excluded. Five more subjects had blood pressures exceeding 180/100 mmHg and were also excluded. The remaining 124 adult donors (72 men, 52 women) fulfilled international blood donation criteria. The interview covered the same questions as outlined above. Before blood donation, blood pressure and heart rate were measured with an automatic digital manometer (Dinamap, Criticon). The total body surface was calculated by the Du Bois formula: total body surface = 0.007184 × weight0.425 × height0.725. At the end of the donation period, 5 mL of blood was withdrawn into EDTA-containing tubes.

Statistical methods. For statistical analysis of paired data, we used the Wilcoxon matched-pairs signed-rank test. The 95%-confidence interval was calculated as the mean ± 1.96 times the standard deviation. For the determination of various influences (age, blood pressure, heart rate) on ANF and cGMP, a multivariate regression analysis was carried out (SPSS statistical software, 1986 ed.; Microsoft Corp., Cary, NC).

Results

Protocol I. Table 1 summarizes the main characteristics of the group investigated in Protocol 1. In the 27 volunteers, the mean time for blood donation, and thus mean
time between first and second sample withdrawals, was 380 s. During this 6.5-min interval, there was a statistically significant decrease in hemoglobin and hematocrit values, and in erythrocyte count. Mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration remained unchanged.

We observed a significant decrease in diastolic blood pressure and mean arterial blood pressure. Even so, the decrease of systolic blood pressure was not yet statistically significant (P = 0.0506).

Interestingly, ANF concentrations in plasma increased from 78.9 (SEM 12.7) to 87.4 (SEM 11.1) ng/L (Figure 1A). This change was statistically significant (P = 0.0035) and independent of age and basal ANF values. There was no correlation between the change of ANF and the duration of blood donation.

Blood donation had no significant influence on cGMP concentrations: 4.6 (SEM 0.6) nmol/L vs 4.5 (SEM 0.6) nmol/L (Figure 1B).

Protocol II. Table 2 lists the main characteristics of the subjects investigated in Protocol II. The mean age of the 124 healthy blood donors was 39.9 (SD 11.95) years. The number of individuals in four age groups (20–30, 31–40, 41–50, and >50 years) was similar. Values for ANF ranged from 18 to 151 ng/L (mean 69.5, SD 26.05 ng/L) and were almost normally distributed (Figure 2A). When corrected for the influence of blood donation, the 95% confidence interval for ANF was 10.0 to 112.1 (mean 61.5) ng/L.

The mean cGMP concentration was 2.90 (SD 1.45) nmol/L, 95% confidence interval 0.04 to 5.75 nmol/L (Figure 2B).

Multiple-regression analysis revealed no significant influence of age, heart rate, or blood pressure on the concentration of either ANF or cGMP in plasma.

Values for ANF and cGMP were also independent of body weight, height, and total body surface. Nor could we find any statistically significant correlation between ANF and cGMP concentration in plasma taken from these 124 healthy volunteers at rest.

The males and females in this group differed in respect to height, weight, blood pressure, and heart rate. The men were taller and heavier and had a significantly higher blood pressure than women: mean arterial pressure for men was 103.7, SD 11.06, vs 98.5, SD 10.29, mmHg for women. The women had a statistically significant higher heart rate: 84.4, SD 13.24, vs 78.0, SD 14.10, beats per minute.

Comparing ANF and cGMP concentrations of 72 male

![Fig. 1. ANF (left) and cGMP (right) before and after blood donation](image1)

The change in plasma ANF concentration at the end of the blood donation period is statistically significant, whereas cGMP values remain unchanged.

![Fig. 2. The influence of age on ANF (left) and cGMP (right) concentrations in plasma](image2)

There were no statistically significant differences of ANF or cGMP plasma concentrations in the four age groups.

### Table 1. Blood Pressure and Hematological and Biochemical Measurements In 27 Blood Donors before and after Blood Donation

<table>
<thead>
<tr>
<th>Units</th>
<th>Mean (SEM) Before</th>
<th>Mean (SEM) After</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hct L/L</td>
<td>0.450 (0.015)</td>
<td>0.443 (0.014)</td>
<td>0.0437</td>
</tr>
<tr>
<td>Hb g/L</td>
<td>151.0 (4.8)</td>
<td>148.0 (4.8)</td>
<td>0.0312</td>
</tr>
<tr>
<td>EC T/L</td>
<td>5.11 (0.14)</td>
<td>4.97 (0.14)</td>
<td>0.002</td>
</tr>
<tr>
<td>MCV fl</td>
<td>88.06 (1.37)</td>
<td>89.14 (1.36)</td>
<td>n.s.</td>
</tr>
<tr>
<td>MCH pg</td>
<td>29.95 (0.50)</td>
<td>29.98 (0.49)</td>
<td>n.s.</td>
</tr>
<tr>
<td>MCHC g/L</td>
<td>336.25 (3.94)</td>
<td>334.02 (1.72)</td>
<td>n.s.</td>
</tr>
<tr>
<td>RRd mmHg</td>
<td>91.7 (4.32)</td>
<td>88.3 (3.85)</td>
<td>0.0409</td>
</tr>
<tr>
<td>RRm mmHg</td>
<td>147.9 (6.29)</td>
<td>138.3 (6.04)</td>
<td>0.0506</td>
</tr>
<tr>
<td>MAP mmHg</td>
<td>110.4 (4.7)</td>
<td>103.6 (4.1)</td>
<td>0.0208</td>
</tr>
<tr>
<td>ANF ng/L</td>
<td>78.9 (12.66)</td>
<td>87.4 (11.09)</td>
<td>0.0035</td>
</tr>
<tr>
<td>cGMP nmol/L</td>
<td>4.6 (0.6)</td>
<td>4.5 (0.6)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

* Wilcoxon matched-pairs signed-rank test.

Hct = hematocrit, Hb = hemoglobin, EC = erythrocyte count, MCH = mean corpuscular volume, MCHC = mean corpuscular hemoglobin concentration, RRd = diastolic blood pressure, RRm = systolic blood pressure, MAP = mean arterial pressure, n.s., not significant (P > 0.05).

### Table 2. Various Physical and Biochemical Data for 124 Healthy Blood Donors

<table>
<thead>
<tr>
<th>Age, y</th>
<th>Range</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>Range</td>
<td>SD</td>
</tr>
<tr>
<td>39.9</td>
<td>20–64</td>
<td>11.95</td>
</tr>
<tr>
<td>1.724</td>
<td>1.56–1.92</td>
<td>0.0744</td>
</tr>
<tr>
<td>71.7</td>
<td>50–97</td>
<td>10.82</td>
</tr>
<tr>
<td>1.84</td>
<td>1.52–2.27</td>
<td>0.16</td>
</tr>
<tr>
<td>138.9</td>
<td>106–177</td>
<td>15.17</td>
</tr>
<tr>
<td>82.8</td>
<td>55–100</td>
<td>10.45</td>
</tr>
<tr>
<td>101.5</td>
<td>72–125.7</td>
<td>11.00</td>
</tr>
<tr>
<td>79.8</td>
<td>52–141</td>
<td>14.32</td>
</tr>
<tr>
<td>69.5</td>
<td>18–151</td>
<td>26.04</td>
</tr>
<tr>
<td>2.90</td>
<td>0.4–8.8</td>
<td>1.45</td>
</tr>
</tbody>
</table>

Table 2. Various Physical and Biochemical Data for 124 Healthy Blood Donors

| TBS | Total body surface, HR = heart rate; other abbreviations in Table 1. |

CLINICAL CHEMISTRY, Vol. 35, No. 7, 1989 1521
and 52 female volunteers, we could observe no significant differences. Values for ANF were 68.5 (SD 23.62) ng/L in men and 70.8 (SD 29.26) ng/L in women; cGMP concentrations were 2.96 (SD 1.45) and 2.83 (SD 1.43) nmol/L, respectively.

Discussion

The reason for the increase in ANF within 6.5 min of blood donation is unclear. The work of Sonnenberg and Veress (23) led to the speculation that catecholamines may mediate ANF release. However, recent data show that a high concentration of circulating catecholamines does not increase ANF concentrations in plasma of healthy people (24). In the same individuals a significant drop in blood pressure had no influence on ANF concentrations. The stimulation of the autonomic (sympathetic) nervous system in hypertensive patients even induced a reduction of ANF values (25). Therefore it seems unlikely that either increased catecholamine concentrations in plasma, or a slightly reduced cardiac output followed by decreased renal blood flow, could increase circulating concentrations of ANF.

According to our present knowledge, cGMP follows increasing ANF concentrations in plasma almost immediately. A study of volume loading as a stimulus to increased plasma ANF concentrations showed that a cGMP maximum occurs approximately 10 min after the ANF peak (26). Thus, the time of 6.5 min between sample collection may be too short to detect a cGMP increase in response to increased plasma ANF. However, the exact delay between ANF release and a consequent increase in the circulating concentration of cGMP in plasma remains to be determined.

In accordance with reports from other authors (18, 19, 27), we found no correlation between ANF concentrations and blood pressure in 124 normotensive healthy individuals. This again confirms that, within a normal range, blood pressure per se does not determine ANF release. These findings do not contradict data from other workers who have found increased ANF concentrations in plasma from patients with hypertension (12–16). Long-lasting hypertension leads to left ventricular hypertrophy and, concomitantly, to a decreased left ventricular compliance, followed by increased atrial pressure. Apparently, these morphological changes in the heart, rather than increased blood pressure alone, cause increased circulating concentrations of ANF (15, 28).

Several authors (19–21) have found increased ANF values in the elderly by comparing young and old healthy individuals. However, this approach is not appropriate for investigating the influence of age on biochemical variables. Mortality morbidity increases with age—a factor that such methods are not able to eliminate. Other authors report a significant positive correlation of ANF with age, sometimes only in normotensive subjects (13, 16).

In our group of 124 healthy normotensive subjects, the number of individuals in four different age groups was similar. We found no influence of age on ANF concentration. Again, it is not age per se that determines ANF concentrations in plasma, but rather age-related morphological changes, e.g., in the heart.

Although the 72 male and 52 female subjects differed in respect to weight, height, total body surface, blood pressure, and heart rate, plasma ANF concentrations were similar in both groups. This internal consistency again confirms that these factors have no influence on plasma ANF concentration.

To our knowledge, the influence of age, blood pressure, heart rate, and sex on cGMP, the second messenger of ANF, has not yet been investigated. As with ANF, we found no correlation between cGMP in plasma and blood pressure, age, or heart rate. cGMP values did not differ significantly between males and females.

A tight correlation of ANF with cGMP has been found in patients with various heart diseases (9–11). However, we found no such correlation in a group of 124 healthy subjects at rest.

ANF concentrations in plasma vary considerably during the day, but do not show an actual detectable diurnal rhythm (19). We found similar results for cGMP in patients with mitral valve prolapse and healthy controls (Wencker et al., manuscript in preparation). Thus, at least three factors render the detection of a correlation between ANF and cGMP in vivo difficult: (a) the high intra-individual variations of both ANF and cGMP, (b) the delay between ANF release and cGMP increase, and (c) the different half-life of these biochemical variables in plasma. We conclude that, within the normal range of ANF and cGMP, seemingly stochastic fluctuations of their concentrations in plasma occur and mask the correlation. When ANF concentrations in plasma are increased in patients because of congestive heart failure or by physical exertion, and a broader range of values is taken into account, a correlation can again be observed.

This should be kept in mind when a comparison of samples collected on different days from subjects at rest is intended. Because of the considerable individual variations, one should always use the mean of several consecutive measurements to increase the comparability of values from one patient.

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Determination of cholesterol has recently gained addi-
tional importance after the National Institutes of Health 
Consensus Conference defined cutoffs for cholesterol 
concentration and advocated vigorous treatment by diet 
modification, with possible drug therapy for all patients 
deemed at significant risk for developing coronary heart 
disease (1). The National Cholesterol Education Program’s 
Expert Panel classified cholesterol ≥2.40 g/L as high, 
between 2.00 and 2.39 g/L as borderline-high, and <2.00 
g/L as desirable (2). For physicians to effectively use cho-
lesterol as a predictor for coronary heart disease, 
the methods for quantifying cholesterol must be accurate (3).

Several enzymatic methods for cholesterol demonstrate a 
bias as compared with the Reference Method performed at the 
Centers for Disease Control (4–6). Many enzymatic 
methods for cholesterol are calibrated, evaluated, and 
standardized with lyophilized serum pools. In a previous study, 
results determined with the aca for lyophilized survey 
material (distributed by the College of American Patho-
gists) were lower than results determined with the SMAC 
and RA-1000, whereas results determined with the aca for 
fresh samples of human serum were higher than those 
determined with the SMAC and RA-1000 (5). The average 
value for cholesterol in the Standard Reference Material 
(SRM) 909 (from the National Institute of Standards and 
Technology), as determined with the aca, was 1.32 g/L,