Optimization of the assay for sialic acid determination in low density lipoprotein

Igor A. Sobenin, Vladimir V. Tertov, and Alexander N. Orekhov
Institute of Experimental Cardiology, Russian Cardiology Research Center, Moscow, and Institute for Atherosclerosis Research, Ltd., Moscow, Russia

Abstract  Sialic acid level in blood plasma and circulating glycoproteins is considered to be a marker for a number of pathologic conditions, including atherosclerosis, cancer, etc. The precise measurement of sialic acid level is an important laboratory procedure to allow correct interpretation of results. Colorimetric methods commonly used for the measurement of sialic acid are not highly specific, as interfering substances may alter the results. Among these, malondialdehyde and other aldehydes play the decisive role. In the circulation, aldehydes are commonly produced during lipid peroxidation in the lipid core of lipoprotein particles, especially low density lipoprotein (LDL). To establish the impairment to the sialic acid determination in LDL introduced by interfering substances, the optimized assay based on Warren’s traditional method was developed and tested in 606 LDL samples. The optimization implies the comparison of color developed using the standard Warren procedure with that due to contaminating agents, mainly thiobarbituric acid-reactive substances (TBARS).

In LDL stored at 4°C, the estimates obtained by the modified procedure were 41.5% or 30.1 nmol/mg lower, on average, compared to the standard procedure (n = 45, P < 0.0001). Even in LDL stored at −70°C, sialic acid estimates obtained by the modified procedure were 6.6% or 3.6 nmol/mg lower, on average, compared to the standard measurement (n = 561, P < 0.005). Thus, the modified procedure avoids significant distortion of the measurement induced by the presence of interfering agents.—Sobenin, I. A., V. V. Tertov and A. N. Orekhov. Optimization of the assay for sialic acid determination in low density lipoprotein. J. Lipid Res. 1998. 39: 2293–2299.

Supplementary key words  low density lipoprotein • sialic acid measurement • thiobarbituric acid • resorcinol

In recent years, the data on the presence of sialic acid-poor low density lipoprotein (LDL) in human blood are rapidly accumulated. Several years ago it was demonstrated that LDL from patients with angiographically assessed coronary atherosclerosis, unlike LDL from healthy subjects, induced lipid accumulation in cultured human intimal cells; this effect was called atherogenicity (1). At that time the sialic acid content was the only statistically significant difference between LDL from coronary athero-sclerotic patients and healthy subjects (2). Thus, it was proposed that LDL sialic acid level might play a role in LDL atherogenicity, and a number of experimental studies provided a considerable background for such an assumption (3–5). The high atherogenic potential of sialic acid-poor LDL led to an increasing interest in this type of LDL modification, and several studies were performed to assess the clinical significance of a low LDL sialic acid level (6, 7). Therefore, the precise sialic acid measurement in LDL becomes very important for the correct interpretation of such results. There are relatively few methods commonly used for the measurement of sialic acid levels; the most popular are the thiobarbituric acid (TBA) assay of Warren (8) and the resorcinol method of Svennerholm (9). These colorimetric methods based on the formation of chromophores are not highly specific, and some interfering substances might crucially alter the results of measurement and lead to erroneous conclusions. Among such substances are malondialdehyde (MDA) produced during lipid peroxidation processes occurring in the lipid core of LDL particle and fructose released from non-enzymatically glycosylated proteins. To analyze the influence of these substances, we have developed a modification of the semi-micro thiobarbituric acid-based assay for sialic acid determination in LDL, i.e., a modification of Warren’s method.

MATERIALS AND METHODS

Reagents

N-acetylneuraminic acid (NANA) was purchased from Calbiochem Corp. (La Jolla, CA); d-galactose was from Serva Feinbiochemica (Heidelberg, Germany); d(-)-fructose was from EM Science (Gibbstown, NJ); cyclohexanone was from Fluka AG (Buchs, Switzerland); and all other reagents were from Sigma Chemical Co. (St. Louis, MO).

Abbreviations: apo, apolipoprotein; LDL, low density lipoprotein; TBA, thiobarbituric acid; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substances.

1To whom correspondence should be addressed.
Sialic acid measurement

LDL sialic acid content was measured in 471 subjects during a health screening study as well as in 112 type 1 and type 2 diabetic patients and in 23 hyperlipidemic subjects.

Venous blood (15 ml) was taken from subjects after an overnight fast into plastic tubes containing 1 mm ethylenediamine tetraacetate (EDTA). Plasma was separated by centrifugation (20 min at 900 g), and LDL (1.025–1.063 g/ml) was isolated by a fast two-step preparative ultracentrifugation as described earlier (10). LDL preparations were dialyzed overnight at 4°C against 2,000 vol phosphate-buffered saline (PBS), pH 7.4, containing 1 mm EDTA, sterilized by filtration (pore size, 0.45 μm), and stored either at −70°C for 1–8 weeks or at 4°C for 1–5 days prior to sialic acid measurement. LDL protein was measured according to Lowry et al. (11) immediately before sialic acid assay.

For sialic acid measurement by the resorcinol method (9), LDL preparation aliquots of 100 μl containing 50–120 μg of LDL protein were taken in triplicate in glass tubes (assay samples) and an additional three aliquots were taken for correction samples. One hundred microliters of 0.2 N H₂SO₄ was added, and the samples were incubated for 1 h at 80°C against blank samples in a Yanako UO-2000 model spectrophotometer (Bausch & Lomb). An alternative way of measurement was the chromophore extraction into 1 ml of butylacetate–butanol 85:15 (vol/vol) was added, and the tubes were vortexed vigorously for 10 sec twice and then centrifuged (7 min at 900 g) for phase separation. Absorbance of the organic phase was determined at 615 nm in quartz 0.4-ml cuvette with 1 cm light path against blank samples.

For the assessment of the effects of interfering substances, d-galactose, d-fructose, d-mannose, d-glucose, d-glucosamine, and d-galactosamine, as well as malondialdehyde were tested in each type of assay in various concentrations.

The TBARS level was measured according to Yagi (12).

Statistical methods

Results are reported as mean ± SEM. Significance of differences was evaluated by one-way ANOVA and two-tailed Student’s paired t-test and was assumed for P values <0.05.

RESULTS

The results of the study are presented in Table 1. In the population-based group, the LDL sialic acid level measured by the modified Warren assay was 6.6% or 3.6 nmol/mg lower, on average, as compared to standard Warren’s measurement. However, this small difference was statistically significant. The conditions of sample storage were of great importance. The above data were obtained in those LDL preparations that were stored at −70°C for 1 to 8 weeks prior to the date of assay. A limited number of samples from the population-based group were stored at 4°C for 1–5 days prior to the assay. In these samples, the corrected sialic acid level was 41.5% or 3.6 nmol/mg lower, on average, as compared to estimates obtained from the standard procedure (Table 1). Thus, the standard measurement of LDL sialic acid levels in two population-based groups could give a rise to the assumption that mean levels differed significantly (at P < 0.0001); but this difference was an artifact, as it disappeared entirely when the measurement was performed in a modified manner (P = 0.274).

In LDL stored at −70°C, the error of sialic acid content

For sialic acid measurement by the resorcinol method, LDL preparation aliquots of 100 μl containing 50–120 μg of LDL protein were taken in triplicate in glass tubes and 900 μl of 5% trichloroacetic acid was added. After mild acidic hydrolysis (7 min in a boiling water bath) the samples were centrifuged (5 min at 900 g) and 0.5 ml of clear supernatant was transferred to other tubes. Five hundred microliters of resorcinol reagent (0.2% resorcinol in 30% HCl with 0.25 mm CuSO₄) was added and the tubes were incubated for 15 min in a boiling water bath. After this, 1 ml of butylacetate–butanol 85:15 (vol/vol) was added and the tubes were vortexed vigorously for 10 sec twice and then centrifuged (7 min at 900 g) for phase separation. Absorbance of the organic phase was determined at 544 nm, 584 nm in black 96-well fluororeader plates in the Labsystems Fluoroscan II model fluororeader (Labsystems OY, Finland). The readings of correction samples were subtracted from those of assay samples, thus corrected readings were obtained. Further calculations of sialic acid content in the sample were performed according to calibration curve that was exactly linear within the given range.

### Table 1. Sialic acid content of LDL determined by two methods

<table>
<thead>
<tr>
<th>Group</th>
<th>Storage Conditions</th>
<th>Sialic Acid nmol/mg LDL protein</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Warren’s Method</td>
<td>Modified Warren’s Method</td>
</tr>
<tr>
<td>Population-based</td>
<td>−70°C</td>
<td>48.4 ± 0.9</td>
<td>44.9 ± 0.8</td>
</tr>
<tr>
<td>Population-based</td>
<td>+4°C</td>
<td>70.4 ± 4.8</td>
<td>40.3 ± 2.7</td>
</tr>
<tr>
<td>Diabetic patients</td>
<td>−70°C</td>
<td>30.7 ± 0.5</td>
<td>25.8 ± 0.6</td>
</tr>
<tr>
<td>Hyperlipidemic subjects</td>
<td>−70°C</td>
<td>59.0 ± 3.0</td>
<td>43.9 ± 2.6</td>
</tr>
</tbody>
</table>
determination (the difference in estimates between non-modified and modified assays) did not exceed 10% in 83% of the samples, and only in 5% samples did the error exceed 30% of the corrected level (Fig. 1, panel B). On the other hand, in LDL stored at 4°C, the error was less than 10% in only 32% of the samples, and in 46% of the cases, the error was greater than 30%. Moreover, under such storage conditions, in 9% of the samples, the difference in estimates between non-modified and modified assays was greater than 2-fold (Fig. 1, panel A).

Taking into account the significant difference between sialic acid levels measured by Warren's method in LDL stored under different conditions, we have studied the effect of LDL storage duration on the error level in sialic acid determination (Fig. 2). In freshly isolated LDL, the levels of sialic acid were similar in spite of the standard or modified method used (33.1 ± 0.1 vs. 32.9 ± 0.1 nmol/mg LDL protein, respectively). In LDL stored for 1 day at 4°C, the estimated levels of sialic acid content increased significantly and the rise continued up to the 7th day. The correction used for the modified assay yielded levels close to the initial level up to the 2nd day of storage. However, at longer storage times (beginning from the 3rd day) both methods gave statistically significant excessive estimates, and even correcting for interfering substances failed to yield the levels similar to the initial observations (Fig. 2). It is important to note that LDL for this experiment was obtained from healthy volunteers and was characterized by a low content of TBARS. During storage, TBARS content increased significantly, and measured TBARS level corresponded well to the difference in sialic acid estimates between standard and modified assays (r = 0.88, P < 0.05). The addition of EDTA, but not butylated hydroxytoluene (BHT), to LDL stored at 4°C prevented the increase of sialic acid estimates up to the 4th day of storage, but at the 7th day even EDTA addition could not prevent an inaccurate measurement (Fig. 2). To investigate whether oxidation may lead to erroneous sialic acid estimates, in vitro oxidized LDL were subjected to standard and modified procedures of sialic acid measurement (Fig. 3). After 1 h of in vitro oxidation, the standard measurement produced extremely high values of absorbance, whereas the modified procedure yielded values close to the initial value up to 8 h of oxidation (Fig. 3). Thus, the error in

Fig. 1. Histogram showing the distribution of errors in LDL sialic acid levels determined by the standard Warren's assay in LDL samples stored unfrozen for 1–5 days at 4°C (panel A) and frozen for 1–8 weeks at −70°C prior to the assay (panel B).

Fig. 2. Graph showing the changes in resultant absorbance level determined by standard (panel A, filled symbols) and modified (panel B, hollow symbols) Warren assay. Sialic acid content was immediately measured in freshly isolated dialyzed LDL preparation obtained from normolipidemic healthy volunteer by two methods (standard and modified Warren's procedure). LDL aliquots of 50 μl containing 150 μg protein were stored at 4°C for 0, 1, 2, 3, 4, and 7 days and then frozen at −70°C to allow simultaneous measurement of sialic acid level. After 7 days, absorbance at 549 nm was determined under standard and modified Warren's procedure as described in Materials and Methods. The data show the mean of three determinations ± SEM; (●), LDL stored without antioxidant addition; (○), LDL stored in the presence of 1 mg/ml EDTA; (•), LDL stored in the presence of 0.1 mm butylated hydroxytoluene. Asterisk (*), the significant rise in absorbance as compared to initial level. Dashed lines, the 95% confidence interval for the initial absorbance level.

Fig. 2. Graph showing the changes in resultant absorbance level determined by standard (panel A, filled symbols) and modified (panel B, hollow symbols) Warren assay. Sialic acid content was immediately measured in freshly isolated dialyzed LDL preparation obtained from normolipidemic healthy volunteer by two methods (standard and modified Warren's procedure). LDL aliquots of 50 μl containing 150 μg protein were stored at 4°C for 0, 1, 2, 3, 4, and 7 days and then frozen at −70°C to allow simultaneous measurement of sialic acid level. After 7 days, absorbance at 549 nm was determined under standard and modified Warren's procedure as described in Materials and Methods. The data show the mean of three determinations ± SEM; (●), LDL stored without antioxidant addition; (○), LDL stored in the presence of 1 mg/ml EDTA; (•), LDL stored in the presence of 0.1 mm butylated hydroxytoluene. Asterisk (*), the significant rise in absorbance as compared to initial level. Dashed lines, the 95% confidence interval for the initial absorbance level.
substances, such as agents on sialic acid measurement, we tested a number of mals (at category of patients is significantly higher than in nor-
dard procedure in hyperlipidemic subjects could easily allow the assumption that LDL sialic acid level in this high level of interfering substances, such as TBARS.

For the initial absorbance level.

Graph showing the effect of LDL in vitro oxidation on re-
sultant absorbance level determined by standard (filled circles) and modified (hollow circles) Warren assay. Sialic acid content was measured in the freshly isolated LDL preparation obtained from normolipidemic healthy volunteer upon oxidation in the presence of 10^{-5} m Cu^{2+} at 37°C. The data show the mean of three determina-
tions ± SEM. Asterisk (*), the significant rise in absorbance as com-
pared to initial level. Dashed lines, the 95% confidence interval for the initial absorbance level.

LDL sialic acid measurement by Warren’s assay is due to the presence of products of oxidation, mainly TBARS.

In the diabetic patients’ group, the LDL sialic acid level measured by a modified Warren assay was 15.6% or 4.9 nmol/mg lower, on average, as compared to standard measurement; the difference was statistically significant (Table 1).

The difference between LDL sialic acid content estimated by two methods was very remarkable for the group of hyperlipidemic subjects. The level measured by the modified Warren assay was 36.0% or 15.2 nmol/mg lower, on average, as compared to standard measurement, the difference being statistically significant (Table 1). It is notable that the samples were stored at −70°C for 1 week till the date of analysis, so the error in sialic acid determination seemed to be due to the initially high level of interfering substances, such as TBARS. Thus, the measurement of LDL sialic acid levels by standard procedure in hyperlipidemic subjects could easily allow the assumption that LDL sialic acid level in this category of patients is significantly higher than in normals (at P < 0.0001), but this tendency was not observed when the measurement was performed in the modified manner (P = 0.71).

For the assessment of the possible effect of interfering agents on sialic acid measurement, we tested a number of substances, such as d-glucosamine, d-galactosamine, d-mannose, d-glucose, d-galactose, d-fructose, and malondialdehyde. Among all substances tested, malondialdehyde was the most effective, forming the colored product upon condensation with thiobarbituric acid. The peak of adsorption was at 446 nm, and molar extinction coefficient for this chromophore was 3.7-fold higher than that for sialic acid, under the same conditions of assay. Among sugars, d-fructose was the only one that gave a negligible but measurable effect. The molar extinction coefficient was 82-fold lower than that for sialic acid.

Taking into account that Svennerholm’s method is widely used for LDL sialic acid determination along with Warren’s method, we tested the possible interference of the same substances on sialic acid measurement using the resorcinol assay. We observed that the molar extinction coefficient for sialic acid itself was 8-fold lower than that obtained by the Warren assay, thus demonstrating that the resorcinol method was considerably less sensitive than the thiobarbituric acid-based assay. Malondialdehyde could form a colored product while reacting with resorcinol, and the molar extinction coefficient was 7.1-fold higher than that for sialic acid under the same conditions of assay. Most prominent, d-fructose also interfered very effectively leading to very high absorption levels. In fact, fructose formed the yellow-brownish chromophore upon reaction with resorcinol with an absorption peak at 465 nm. Even at the wavelength of 615 nm used in the assay for sialic acid measurement, the molar extinction coefficient for fructose was only 1.6-fold lower than that for sialic acid, although the absorption for fructose at 615 nm accounted only for 13% of peak value at 465 nm.

**DISCUSSION**

It has been recently demonstrated that there is an in vivo multiple-modified LDL fraction in human blood. This LDL is characterized by numerous alterations in chemical composition and physical properties. The most remarkable one is low protein- and lipid-bound sialic acid content, i.e., this LDL is desialylated. The LDL sialic acid level, on the whole, reflects modified atherogenic LDL in circulation (2, 13, 14). At present, a number of colorimetric methods for sialic acid measurement are widely used, among them the most popular are Warren’s thiobarbituric acid-based assay, Svennerholm’s resorcinol method, and the resorcinol–periodate method of Jourdian, Deau, and Roseman (15). All these methods can hardly be designated as specific, as some widely spread compounds may interfere and alter the results of measurement. Among these are malondialdehyde and fruc-
tose, and we have shown in this study that they can yield dramatically increased estimates of the total sialic acid content of LDL.

Theoretically, it is possible to avoid the influence of lipid peroxidation products by preliminary delipidation of LDL samples. However, this approach would add a cumbersome step to the procedure, and the levels would characterize not total but protein-bound sialic acid content. The
latter may be of great importance, as the decrease in lipid-bound sialic acid content of LDL may be much more prominent than that of protein-bound sialic acid (16). Usually, the level of malondialdehyde in freshly isolated and dialyzed LDL is negligible as compared to that of sialic acid, and in some cases the formation of an additional chromophore not related to sialic acid itself may be ignored. However, inappropriate storage conditions may lead to the substantial increase in LDL TBARS content. Moreover, atherosclerotic or diabetic patients may be characterized by already increased MDA levels in LDL (17, 18). Taking into account the high molar extinction coefficient for MDA, it would generally be incorrect to disregard the possibility of obtaining erroneously high estimates of sialic acid due to MDA presence.

To clarify the situation with interfering fructose, it may be relevant to discuss briefly the processes of nonenzymatic glycation that occur in the circulation in all subjects. During nonenzymatic glycation, sugars act in a time- and concentration-dependent manner, glycating α-amino groups of lysine and N-terminal amino acids. The first glycation product, glycosylamine or Schiff base, undergoes an Amadori rearrangement over a period of few days and yields the more stable ketoamine or Amadori product. The latter is a β-ketoamine existing both in pyranose and furanose ring forms, and during mild acidic hydrolysis a significant part of Amadori products is released as fructose-resembling furanose derivatives (19). Colorimetric methods do not possess strict specificity and therefore cannot distinguish the contribution of sialic acid and fructose in the formation of chromophore. The influence of furanose derivatives increases dramatically in hyperglycemic subjects, in whom the glycation of proteins can exceed normal values up to 4-fold (20, 21). Thus, in diabetic patients it is quite necessary to examine the results of sialic acid colorimetric assays with certain care and consider the possibility of obtaining erroneously high levels. From this point of view, the thiobarbituric acid-based assay is preferable, as molar extinction coefficient for fructose is negligibly low. On the other hand, Svennerholm’s method (as well as other resorcinol-based assays) can hardly be used for LDL sialic acid determination in diabetics, because the molar extinction coefficient for fructose is rather high, and the content of glycation products (generally called fructosamine) is comparable to that of sialic acid (21). Approximate calculations show that the LDL sialic acid estimates obtained by resorcinol assay may exceed real values even by 2.5-fold. Due to these considerations, we have abandoned the use of Svennerholm’s method for LDL sialic acid determination in our laboratory.

The validity of correction introduced into standard Warren’s assay was assessed in our recent work, where carbohydrate content of LDL was determined also by anion exchange chromatography using the pulsed amperometric detection method, and practically coinciding estimates have been obtained (16). Moreover, we have compared the data on LDL sialic acid content obtained in different studies (Table 2) (2, 3, 6, 7, 13, 16, 21–35). For convenience, the values are expressed as mol sialic acid per mol LDL protein. It is known that apolipoprotein B has 20 potential N-glycosylation sites, of which 16 were found to be definitely glycosylated (36). Polysaccharide moiety of human apoB consists of 5–6 mol high-mannose type and 8–10 mol complex (biantennary) oligosaccharides per mol apoB protein (36). As the biantennary type chain can bear two sialic acid residues, the maximum theoretical value for protein-bound sialic acid level may account for 20 mol sialic acid per mol of apoB protein. Additional sialic acid may come from the LDL lipid moiety. In our recent work we have shown that the level of lipid-bound sialic acid in LDL is 2–4 mol per mol of LDL protein (16). Thus, the maximum amount of total sialic acid in native LDL may be estimated as 24 mol per mol of LDL protein. The results of different studies shown in Table 2 are mainly in good agreement with the proposed theoretical value. In our early work (2), the reported results were moderately higher in comparison with other studies, but the correction for sialic acid determination in LDL was not yet introduced, and the group of patients was rather small thus giving high deviation. The same considerations may be suggested for the results obtained by La Belle and Krauss (32). Melajärvi, Gylling, and Miettinen (35) obtained incredibly high values of LDL sialic acid content for healthy subjects and diabetic patients that are in sharp contrast to the data from other studies. The 3- to 15-fold excess of sialic acid as compared with usually observed levels may be explained only by the evident inaccuracy of the resorcinol-based determination method mainly used in that study. The evaluation of LDL sialic acid in the diabetic patients group requires that any resorcinol-based assay should be avoided, as the principle of method itself does not allow us to distinguish between sialic acid and fruronosyl residues. Additionally, the very long isolation

| Table 2. Reference data on sialic acid content of human LDL |
|----------------|----------------|----------------|----------------|
| LD L Sialic Acid Content Ref. No. Authors Year |
| mol/mol LDL protein | 25 | 22 | Schultze and Heide 1960 |
| | 17 | 23 | Ehnholm et al. 1972 |
| | 10 | 24 | Kutterovich et al. 1974 |
| | 19 | 25 | Van der Bijl 1974 |
| | 10 | 26 | Fontaine and Malmendier 1975 |
| | 14 | 27 | Lee and Breckenridge 1976 |
| | 28 | 28 | Swaminathan and Aladjem 1976 |
| | 13 | 29 | Fontaine and Malmendier 1978 |
| | 17–28 | 3 | Filipovic et al. 1979 |
| | 33 | 30 | Shierman and Fisher 1979 |
| | 15–21 | 31 | Maruhama et al. 1983 |
| | 14–45 | 2 | Orekhov et al. 1989 |
| | 39–52 | 32 | La Belle and Krauss 1990 |
| | 8–17 | 13 | Tertov et al. 1992 |
| | 19–27 | 6 | Ruelleand et al. 1993 |
| | 14–21 | 21 | Sobenin et al. 1993 |
| | 9–15 | 16 | Tertov et al. 1993 |
| | 11–18 | 33 | Sobenin et al. 1994 |
| | 6–10 | 34 | Barbosa et al. 1995 |
| | 10–24 | 7 | Chappey et al. 1995 |
| | 66–370 | 35 | Melajärvi et al. 1996 |
| | 13–23 | | this study |
procedure and the series of repeated ultracentrifugations could easily result in abundant accumulation of lipid peroxidation products in LDL that interfere in either method used, resorcinol-based or Warren's. Such a suggestion can easily explain the fact that sialic acid estimates in LDL subdivided into light, dense, and very dense fractions were in any way significantly higher than in total LDL prior to separation by density gradient ultracentrifugation (35). The possibility of erroneous sialic acid determination by resorcinol-based assay was suspected as far back as 1959, when Warren himself reported on the 1.4- to 5.4-fold difference in sialic acid estimates made by thiobarbituric acid and resorcinol methods (8). At that time, the reason for such a discrepancy was not explained, but it can be suggested that sufficient amounts of products of non-enzymatic glycation were present in the biological materials used for comparison (beef brain, beef liver, egg white etc.).

In conclusion, our experience of LDL sialic acid measurement allows us to recommend a modified thiobarbituric acid assay as a relatively easy and inexpensive method. The correction procedure avoids significant distortion of the results induced by the presence of interfering agents, mainly the products of lipid peroxidation. A more precise measurement of LDL sialic acid level would help to evaluate the diagnostic and prognostic significance of this parameter.

The research described in this publication was made possible in part by Grant #588 from Russian Council on Atherosclerosis and Grants #07-04-50124 and 97-04-48461 from Russian Foundation for Basic Research.

Manuscript received 8 January 1998 and in revised form 22 June 1998.

REFERENCES

30. Shierman, R. B., and W. R. Fisher. 1979. The absence of a role for...


