INTERACTION BETWEEN NUCLEIC ACIDS AND BERBERINE SULFATE

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Mellors *et al.* (1) have found by means of microfluorometry that an approximately linear relation exists between the fluorescence intensity of cell nuclei stained with berberine sulfate and their nucleic acid content. However, very little is known as to the interaction between nucleic acids and berberine sulfate *in vitro*. The purpose of the present study is to examine this interaction *in vitro* in the hope of developing a fluorometric method for nucleic acid analysis.

MATERIALS AND METHODS

For fluorometric determination, a 0.25 mM aqueous solution of berberine sulfate (Merck) was used as the reagent. First, a 2 ml aliquot of the reagent was mixed with an equal volume of distilled water, $MgCl_2$ solution, or NaCl–Na citrate solution as described below. This mixture was excited by long wavelength ultraviolet light and its fluorescence intensity was determined. The standard primary fluorescence intensity was 5 in arbitrary units. Then, a mixture of 2 ml of the reagent and 2 ml of the solution to be tested was prepared, excited, and its secondary fluorescence intensity determined. The secondary fluorescence intensity may be enhanced or may remain unchanged from the primary one, depending on the substance tested. The difference in intensity between the primary and the secondary fluorescence, if any, was taken as the "fluorescence increment."

PREPARATION OF RNA: Cells of *Saccharomyces cerevisiae* were ground with sea sand in 0.25 M sucrose solution. Cell debris was removed by squeezing through double layers of muslin. The resulting filtrate was centrifuged at 10,000 *g* for 20 minutes. The supernatant thus obtained was centrifuged again at 104,000 *g* (Spinco model L ultracentrifuge) for 90 minutes to separate microsomal pellets (*cf.* 2). All the fractionation procedures were performed in a cold room at about 5°C. Kirby's phenol method (3), slightly modified by Otaka and Osawa (4, 5), was employed to prepare microsomal RNA (Ms-RNA) from the pellets. Soluble RNA (s-RNA) was generously supplied by Dr. K. Kuriki of the Department of Medical Chemistry, Faculty of Medicine, Kyoto University, who had prepared it by the method of Monier, Stephenson, and Zamecnik (6). The RNA prepared by these methods was dissolved in 2.4 mM $MgCl_2$ solution (pH 5.4). Mg-oligonucleotide was generously supplied by Dr. Y. Sugino of the Department of Medical Chemistry, Faculty of Medicine, Kyoto University, who had prepared it by precipitation in ethanol after the digestion of yeast RNA by RNAase.

PREPARATION OF DNA: DNA was prepared by Dr. Sugino from calf thymus, employing the method of Kay, Simmons, and Dounce (7). The DNA prepared was dissolved in a mixture of equal volumes of 0.3 M NaCl and 0.03 M Na citrate solutions (pH 7.2) (8).

DENATURATION OF DNA: The DNA sample was heated at 98°C for 10 minutes in a mixture of equal volumes of 0.3 M NaCl and 0.03 M Na citrate solutions and subdivided into two aliquots, one being cooled quickly, the other slowly (9, 10). In the former case the sample was immersed in ice water, while in the latter case it was kept in a water bath at 98°C and allowed to cool for 10 hours at room temperature.

OTHER CHEMICALS: Commercial preparations of chondroitin sulfate, of hyaluronic acid, polyphosphoric acid, egg albumin, globulin, protamine, histone, and nucleoside monophosphates were obtained and dissolved in distilled water.

DETERMINATION OF NUCLEIC ACID–P: Allen's method (11) was employed.

APPARATUS: The absorption spectrum was determined with a Hitachi photoelectric spectrophotometer EPU-2. The fluorescence intensity was measured with the same instrument equipped with an attachment for fluorescence photometry L-3. This

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fluorometer was standardized against 0.125 mM berberine sulfate solution to give a fluorescence intensity of 5 when the solution was excited by the Hg line of nearly 365 mμ.

RESULTS

Preliminary Experiments

When a berberine sulfate solution is excited by long wavelength ultraviolet light, it shows a yellow fluorescence having an emission maximum at 540 mμ. First, the effects of the concentration of the berberine sulfate solution, of different pH's, and of coexisting inorganic salts on fluorescence intensity were examined. The primary fluorescence intensity is maximum in 0.125 mM berberine sulfate; it remains constant between pH 1.7 and pH 8.0, and is not affected by coexisting salts, NaCl, Na citrate, and $MgCl_2$. Next, the effect of certain substances, which may be found in cells, on the fluorescence intensity of berberine sulfate was studied. When a quantity of DNA or RNA is added to berberine sulfate solution and the mixture is excited by ultraviolet light, a marked increase of fluorescence intensity at 540 mμ is observed. When hyaluronic acid, polyphosphoric

TABLE I

Fluorescence Increment of Berberine Sulfate–Biochemical Substance Mixtures

Excitation of the mixtures was produced by the Hg line of nearly 365 mμ. The secondary fluorescence intensity of the mixtures was measured at 540 mμ. The standard value of the primary fluorescence intensity of 0.125 mM berberine sulfate solution was 1 in arbitrary units. Final concentration of berberine sulfate in these mixtures was 0.125 mM. The difference in intensity between the primary and the secondary fluorescence was taken as the fluorescence increment. The globulin was dissolved in 0.9 per cent NaCl, but the other substances were in aqueous solutions.

Bio chemical substance	Fluorescence increment
0.015 % s-RNA*	86.5
0.015 % chondroitin sulfate	18.0
0.015 % hyaluronic acid	3.0
0.015 % polyphosphoric acid	0
0.015 % egg albumin	1.0
0.015 % globulin	0
0.015 % protamine	0
0.015 % histone	0
0.5 mM 5′-AMP	0
0.5 mM 5′-UMP	0
0.5 mM 5′-GMP	0
0.5 mM 5′-CMP	0

* Other nucleic acids, namely native DNA and Ms-RNA, were also tested. But in either case, the fluorescence intensity was very strong and beyond the scale.

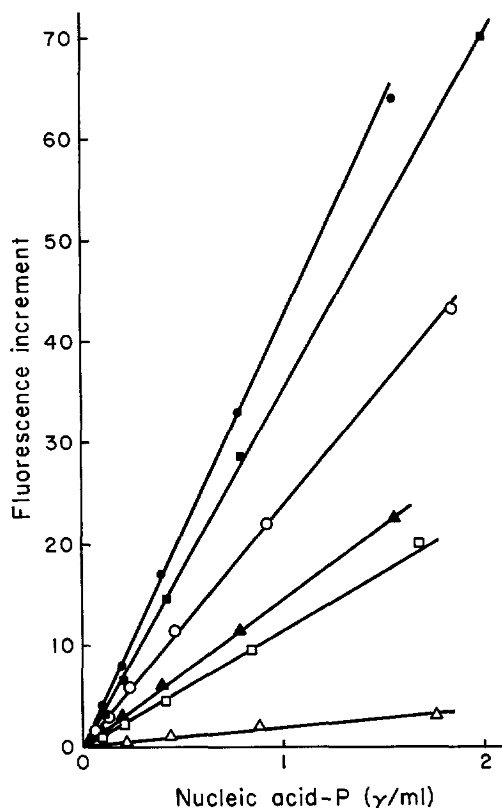

FIGURE 1

Linear relationship between the fluorescence increment of berberine sulfate–nucleic acid or berberine sulfate–Mg-oligonucleotide mixtures and the concentration of nucleic acid or Mg-oligonucleotide. Ordinate refers to the fluorescence increment at 540 mμ. Abscissa refers to the concentration of nucleic acid or Mg-oligonucleotide expressed by γ nucleic acid–P or Mg-oligonucleotide-P per milliliter sample. Open circles, berberine sulfate–Ms-RNA mixture; open squares, berberine sulfate–s-RNA mixture; open triangles, berberine sulfate–Mg-oligonucleotide mixture; solid circles, berberine sulfate–quickly cooled denatured DNA mixture; solid squares, berberine sulfate–slowly cooled denatured DNA mixture; solid triangles, berberine sulfate–native DNA mixture.

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acid, egg albumin, globulin, protamine, histone, or a nucleoside monophosphate is added to berberine sulfate solution, no recognizable increase of fluorescence intensity is observed. However, as shown in Table I, chondroitin sulfate has a slight effect on the fluorescence intensity. Therefore, the fluorescence intensity of berberine sulfate is specifically increased by the addition of nucleic acids, provided that the material to be tested does not contain chondroitin sulfate.

Examination of the absorption spectrum of berberine sulfate solution alone, of RNA–berberine sulfate mixture, and of DNA–berberine sulfate mixture shows that a slight shift of absorption peak to a longer wavelength takes place in the mixtures (*cf.* 12). It seems highly probable, therefore, that a "berberine sulfate–nucleic acid complex" is formed when these two substances are mixed in aqueous solution.

Effect of RNA on the Fluorescence Intensity of Berberine Sulfate

The relation between the fluorescence increment and the RNA concentration of the sample is shown in Fig. 1. It is clear that a linear relationship exists between the amount of Ms-RNA, s-RNA, or Mg-oligonucleotide added and the fluorescence increment caused by these substances. The atomic extinction coefficient values at 260 mμ with respect to phosphorus, *i.e.* E(P), are 7150 in Ms-RNA, 7590 in s-RNA, and 9200 in Mg-oligonucleotide, respectively. This figure indicates, therefore, that the RNA of small E(P) value enhances the fluorescence intensity of berberine sulfate more markedly than does that of large E(P) value. It is noted here that oligonucleotide from RNA causes only a very slight increase of fluorescence intensity.

Effect of DNA on the Fluorescence Intensity of Berberine Sulfate

In this experiment, native DNA, slowly cooled denatured DNA, and quickly cooled denatured DNA were used as DNA samples. As shown in Fig. 1, the fluorescence increment of the berberine sulfate–DNA complex increases linearly with the concentration of DNA, as in the case of RNA. The E(P) value is 7030 in the native DNA, 7600 in the slowly cooled DNA, and 7740 in the quickly cooled DNA. It is noted that the fluorescence increment caused by the quickly cooled denatured DNA is 2.9 times, while that caused by the slowly cooled one is only 2.4 times, that of the berberine sulfate–native DNA complex, provided that an equal amount of DNA is added to the berberine sulfate reagent.

DISCUSSION AND CONCLUSION

In the present investigation, two kinds of RNA of different molecular weights, Ms-RNA and s-RNA, were used. The former has a higher molecular weight than the latter, as is suggested by the fact that yeast s-RNA has a molecular weight of 27,000 and RNA from yeast ribonucleoprotein particles has a molecular weight of 1,100,000 (4, 5). As to the Mg-oligonucleotide used, its E(P) value is far larger than that of the Ms-RNA or s-RNA used. In view of the fact that the extinction coefficient of RNA increases significantly on degradation (13), the Mg-oligonucleotide used is more degraded than the Ms-RNA and the s-RNA. The result obtained in the present study shows that the fluorescence increment of the berberine sulfate–Ms-RNA complex is the largest and that of the berberine sulfate–Mg-oligonucleotide complex is the smallest (Fig. 1). It may be concluded, therefore, that the presence of high molecular weight polyribonucleotides increases the fluorescence intensity of berberine sulfate more strongly than does the presence of low molecular weight polyribonucleotides. This conclusion is also supported by the fact that nucleoside monophosphates do not enhance the fluorescence intensity of berberine sulfate. Oster (14) has stated that the increase of fluorescence intensity of auramine O on the addition of nucleic acids depends on their molecular weight. This view agrees with the conclusion of the present author stated above.

According to Jordan (15), the E(P) value of a DNA sample may be taken as an indication of the grade of denaturation. Among the native and denatured DNA's tested, the ability to enhance the fluorescence, when they are added to the berberine sulfate reagent, is as follows: the quickly cooled denatured DNA (E(P), 7740) > the slowly cooled denatured DNA (E(P), 7600) > the native DNA (E(P), 7030). Therefore, it is not improbable that the fluorescence intensity of the berberine sulfate–DNA complex depends on the degree of heat denaturation, that is, on the structural change of DNA involved (*cf.* 9, 10).

In the present study, it is found that a linear relationship exists between the fluorescence incre-

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ment of the berberine sulfate–nucleic acid complex and the amount of nucleic acid added. This finding may be used to develop a new fluorometric method for the quantitative determination of nucleic acids. It is to be emphasized here that this finding is consistent with the results of Mellors *et al.*, who studied the berberine-stained cell nucleus *in situ* (1).

SUMMARY

Though an aqueous berberine sulfate solution excited by long wavelength ultraviolet light manifests only a weak fluorescence, it shows an intense yellow fluorescence on the addition of a small quantity of RNA or DNA. This enhancement of yellow fluorescence is larger when microsomal RNA is used as an additive than when low molecular weight RNA is used. The addition of heat-denatured DNA markedly increases the fluorescence intensity as compared with that of native DNA. In either case, a linear relationship exists between the fluorescence increment and the amount of nucleic acid added to the berberine sulfate solution. From these results, the possibility of developing a new fluorometric method for nucleic acid determination is suggested.

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REFERENCES

1. Mellors, R. C., Glassman, A., and Papanicolaou, G. N., *Cancer*, 1952, **5,** 458.
2. Ichimura, K., Izawa, M., and Oota, Y., *Plant and Cell Physiol.*, 1960, **1,** 317.
3. Kirby, K. S., *Biochem. J.*, 1956, **64,** 405.
4. Otaka, E., and Osawa, S., *Nature,* 1960, **185,** 921.
5. Osawa, S., *Biochim. et Biophysica Acta,* 1960, **43,** 110.
6. Monier, R., Stephenson, M. L., and Zamecnik, P. C., *Biochim. et Biophysica Acta,* 1960, **43,** 1.
7. Kay, E. R. M., Simmons, N. S., and Dounce, A. L., *J. Am. Chem. Soc.*, 1952, **74,** 1724.
8. Doty, P., Boedtker, H., Fresco, J. R., Haselkorn, R., and Litt, M., *Proc. Nat. Acad. Sc.*, 1959, **45,** 482.
9. Marmur, J., and Lane, D., *Proc. Nat. Acad. Sc.*, 1960, **46,** 453.
10. Doty, P., Marmur, J., Eigner, J., and Schildkraut, C., *Proc. Nat. Acad. Sc.*, 1960, **46,** 461.
11. Allen, R. J. L., *Biochem. J.*, 1940, **34,** 858.
12. Yamagishi, H., *Cytologia,* 1962, in press.
13. Davidson, J. N., The Biochemistry of the Nucleic Acids, London, Methuen, 1960.
14. Oster, G., *Compt. rend. Acad. sc.*, 1951, **232,** 1708.
15. Jordan, D. O., The Chemistry of Nucleic Acids, London, Butterworth, 1960

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