Reports and Letters

New Color Test for Thiols and Thiolesters

In the course of experiments with homocysteine peptides (*1*), it was discovered that, when the products of the reaction between N-ethyl maleimide (NEM) and thiols are made alkaline, a red color develops (*2*). Under the proper conditions, this reaction is extremely sensitive. The color is much more stable than that given with nitroprusside, and, in contrast to the iodine-azide reaction, it is not given by disulfides or thioethers.

This reaction, the mechanism of which is still obscure, shows a number of interesting peculiarities: (i) The color is unstable in the presence of water, probably owing to hydrolysis of the imide ring. It is much more stable in ethanol and isopropanol but not in methanol. (ii) The color is an acid-base indicator, since it is discharged by acid and reappears upon the addition of alkali. (iii) The maximum color is not produced with equimolar concentrations of thiol and NEM. It is greatly potentiated by an excess of NEM. Thus $10^{-5}M$ concentrations of sulfhydryl can easily be detected but only if the NEM concentration is of the order of $0.1M$. (iv) The color is not produced when the NEM is exposed to alkali prior to the addition of thiol. This is undoubtedly due to the instability of NEM in alkaline solution (*3*).

It is interesting to note that Piutti and his collaborators (*4*) found many years ago that hydroxides and alcoholates of alkali metals produced violet colors with unsaturated imides—for example, N-methyl maleimide, N-ethyl maleimide, and N-benzyl maleimide. It must be stressed, however, that the color reaction with thiols is several orders more sensitive than that with alcohols, so that interference by alcohols can be eliminated.

The new color reaction (*5*) provides an excellent means of visualizing sulfhydryl compounds on paper. It is especially suitable in connection with the systems for the separation of thioamino acids and peptides in the form of their NEM derivatives, which were described previously (*6, 7*). The main advantage of this method is the stabilization of the thiols during chromatography. However, since the usual color reactions for sulfhydryl groups are not given by the NEM complexes, ninhydrin had to be used to stain the chromatograms.

The new color reaction makes it possible to combine the advantage of protecting the sufhydyryl group during chromatography with that of visualizing the compounds by a reaction specific for this group. In this way, sulfhydryl-containing amino acids and peptides can be distinguished from other amino acids and peptides of the same R_f, which would not be possible with ninhydrin. Moreover, chromatography of thiols, as their NEM derivatives, can now be extended to thiols that do not contain an amino group. The following procedure is recommended for the visualization of thiols or thiolesters.

Following chromatography by the methods described previously (*7*), the paper is dried thoroughly in a current of air. It is then dipped in a $0.05M$ solution of NEM in absolute isopropanol. The paper is again dried in a current of air for 15 minutes, followed by dipping in $0.25M$ potassium hydroxide (KOH) in absolute isopropanol. Pink to red spots appear immediately.

No color reaction is observed if the order of dipping is reversed or if a chromatogram treated with NEM is left to dry for 2 days before treatment with KOH. Isopropanolic solutions that contained higher and lower concentrations of NEM (0.1 to $0.025M$) and of KOH (0.5 to $0.125M$) were found adequate for dipping purposes. However, using the lower NEM concentration reduces the sensitivity of the method somewhat, whereas the higher concentration leaves a light pink background on the paper. This is undoubtedly caused by the Piutti reaction (*4*). In the case of NEM this background reaction is not observed with the recommended concentration, probably because of the volatility of this particular maleimide. On the other hand, a distinct red background is observed with N-phenyl maleimide, and the use of N-ethyl maleimide is therefore strongly recommended for paper chromatography.

The isopropanolic NEM solution was found to be stable for at least 8 days if it was stored in a dark glass container in the refrigerator. The color reaction was used successfully after chromatography with the following solvent systems: isopropanol-formic acid-water, *n*-propanol-water, *n*-butanol-formic acid-water, isopropanol-ethanol-formic acid-water (*7*). Identical R_f values were obtained for compounds containing both an amino and a sulfhydryl group with NEM/KOH and with ninhydrin.

As little as 1 μg (0.033 μmole) of glutathione, or 0.1 μg of sulfhydryl could be detected. Concentrations of this magnitude faded after a few hours. Higher amounts, 3 μg and above, were stable for more than 48 hours. The color reaction was positive in solution and on paper with the following compounds: hydrogen sulfide, methyl mercaptan, ethyl mercaptan, amyl mercaptan, thioglycolic acid, ethyl thioglycolate, thioacetic acid, thiomalic acid, thiouracil, ergothioneine, cysteine, cysteinyl-glycine, glutamyl-cysteine, glutathione, coenzyme A; 2,3-dimercaptopropanol (BAL), lipoic acid, S-acetyl thioglycolic acid, S-acetyl glutathione, homocysteine thiolactone, and N-acetyl homocysteine thiolactone. Alcoholic solutions of ovalbumin also gave a pink color with NEM and alkali.

It is particularly interesting that bacitracin gave a positive reaction, since this peptide fails to show any of the common tests for sulfhydryl groups except after hydrolysis (*8*). No color reaction was obtained with the following: glycine, glutamic acid, glutamine, tyrosine, alanine, serine, aspartic acid, asparagine, valine, methionine, threonine, tryptophan, phenylalanine, dihydroxyphenylalanine, proline, cystine, homocystine, cystinyl-diglycine, diglutamyl-cystine, or oxidized glutathione (GSSG).

This reaction should serve as a useful tool for distinguishing and detecting sulfhydryl compounds, especially those in which the thiol group is the only functional group in the molecule. This is illustrated by our experience with some preparations of cysteinylglycine, which were found by this method to contain mercaptan impurities that do not give a ninhydrin reaction.

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References and Notes

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Radioactivity in Thyroid Glands Following Nuclear Weapons Tests

Radioactivity has been reported (*1*) and confirmed (*2*) in the thyroid glands of cattle, presumably from I^{131} fallout. The present report (*3*) is a continuous study of the radioactivity in thyroids from the United States, Canada, England, Germany, and Japan during the past year. There was a series of 14 nuclear tests in Nevada from 18 February to 15 May 1955 and a test in the eastern Pacific Ocean in mid-May 1955 (*4*). Additional sources of fission products were presumably released by other countries in the winters of 1954 and 1955.

Dunning (*5*) has estimated biological ingestion of iodine fission products beginning 50 hours after fission results in more than 80 percent of the total radiation dose to the thyroid being due to I^{131}. When ingestion begins later, I^{131} rapidly becomes a more dominant iodine isotope. In the present investigation (*6*) all the radioactivity reported in thyroid glands will be considered as I^{131}.

The simple counting methods described previously (*1*) were used, except that after June 1955 all samples that contained less than 0.002 mμc/g were tested in a 1¾ by 2-in. NaI(Tl) well crystal in conjunction with a pulse height analyzer to increase accuracy in the determination. All samples were counted with a coefficient of variability (*7*) ±5 to 12 percent, except those between October 1954 and June 1955 that contained less than 0.01 mμc/g; in these the coefficient of variability was ±30 to 50 percent. The instruments were calibrated daily against an I^{131} standard. The well crystal with pulse height analyzer had a background of 0.12 count/sec and 1μc I^{131} counted 6600 count/sec.

Human thyroids from all available autopsies in Memphis were tested, and the results from all 175 glands have been shown in Fig. 1. Thyroids from 15 unselected slaughterhouse cattle raised within 200 miles of Memphis were tested each week for 70 weeks. During the period of greatest radioactivity five to ten thyroids were received by airmail every 1 or 2 weeks from England, Germany, Canada, Washington State and every 2 or 3 weeks from Japan. The radioactivity of these glands showed standard deviations similar to those from Memphis; therefore, for simplicity, only the average values for the foreign glands have been presented. The radioactivity was corrected for decay during the 4 to 10 days spent in transportation. The millimicrocuries per gram were plotted against the date of slaughter and bar graphs (Fig. 2) were constructed from these curves. Only the data for the first and 15th of each month are included in Fig. 2, and no bar is shown for an area unless there was a sample within 1 week of the date.

On 17–19 April 1955 the gamma radioactivity in the thyroid area was determined *in vivo* in 20 persons (USPH monitors and their families in Utah and Nevada). Most of these subjects were men stationed around the nuclear weapons tests site. Their work was to determine fallout patterns. A scintillation crystal was placed against the thyroid areas of their necks, and the results were compared with determinations over the thigh. Precautions were taken to prevent errors owing to surface contamination. The limit of sensitivity of these *in vivo* determinations was 2 mμc I^{131}. The results showed that only two individuals (men) had detectable amounts of radioactivity in their thyroid areas, and each of these has a total gamma-emitting equivalent of approximately 5 mμc I^{131}.

The relationship between total chemical iodine and radioactive iodine in cattle thyroids was investigated by analyses of 20 glands with greatest extremes in I^{131} content. These showed no correlation between I^{127} and I^{131} content.

Figure 1 summarizes the most complete data. The ordinate is plotted on a 5-cy log scale in order to show early increases from the base line and also include the maxima, 10,000 times greater. The maximum value in any sample group was frequently 10 times greater than the minimum collected at the same time. Yet, every time the average value increased above 0.002 mμc/g, the minimum was observed to increase. This suggested that the minimum intakes were possibly due to some mechanism, such as respiration, common to all the animals. The maximum values may be dependent on additional variable factors, such as ingestion of fallout material.

The increases shown in October 1954 to 15 February 1955 and November 1955 to March 1956 were believed to be due to nuclear tests, which, so far as I am aware, were not conducted by the USAEC.

Five days after the first test of the Nevada series, there was a detectable increase in the I^{131} content of cattle thyroids in Memphis. The minimum was above minimum detectable level and did not return to that level for 8 months. The observed rate of increase of thyroid I^{131} may be sensitive to the frequency of sampling and the freedom permitted the animal 2 or 3 days preceding slaughter. Two weeks elapsed between the beginning of the Nevada series and the first maximum of that period. The last maximum, 6 June, was observed 3 weeks after

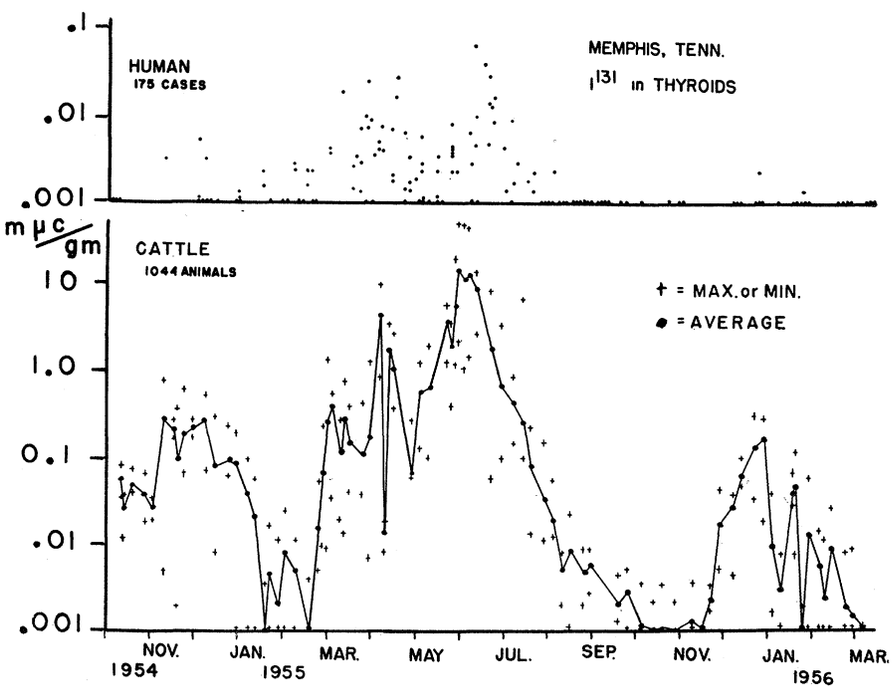

Fig. 1. Iodine-131 in human and cattle thyroid glands.