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A PASTEUR PROBE

A Proposed Experiment

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"Cytochemical Studies of Planetary Microorganisms

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The significance of optical activity for the recognition of life is too well known to require further amplification. As documented in the attached paper by Halpern and Westley, a method is available whereby important metabolites like amino acids can be scanned for optical activity with very high sensitivity, the detection of 100 nanograms being rather easily accomplished (not unreasonably sensitivities down to 1 nanogram should be achievable within the general state of the art). This method depends on the coupling of an optically active reagent, such as L-N-trifluoroacetyl-prolyl chloride, to the amino acid ester. If the amino acid is racemic, two diastereoisomers, the L-D and the L-L dipeptides will be formed, and these often prove to be readily resolvable by gas chromatography. The reactions involved are quite straightforward, run smoothly and quantitatively, and can be automated quite readily. The same approach should be easily generalized to other optically active species, organic acids generally, as well as alcohols and amines, and is being explored accordingly, especially for applications to carbohydrates. Besides their abundance and multifarious functions in the cell, carbohydrates have the advantage that methods are available whereby they can be degraded to a unique asymmetric compound. This would make it possible to test the whole genus of carbohydrates for net optical activity without needing to specify exactly which sugar is in question.

The work with amino acids does point to this limitation, namely that if a wide variety of organic molecules are present in the sample, the gas chromatograph would not be easily interpretable, since any two peaks might be related diastereoisomers, or totally unrelated molecules. This difficulty could be circumvented in principle in several ways:

a. a two-stage separation, the first without the introduction of an optically active probe - for example, trifluoroacetylation; then each fraction would be tested by resolvability, using D and L reagents separately and together prior to the second stage. This is clumsy and may be difficult to implement without racemization.

b. a single stage reaction run in parallel with D- and L- reagents. This may suffer from calibration problems circumvented by (c).

c. a single stage reaction with ratio-detection of D- and L-complexes. For example, suppose we prepared the enantiomeric reagents with differential labels, for sake of argument say levo-tritium and dextro-C^{14}. After coupling

to the mixed target material, the product is then chromatographed. For each symmetrical target molecule, the $^{3}H/^{14}C$ ratio will remain uniform in a single peak. However, if an optically active asymmetric molecule is encountered, and gives rise to resolvable diastereoisomers, the tritium will be concentrated in one peak, the C^{14} in another; that is to say there will be a swing in the ratio of the labels to one another. If the target molecule is racemic, two peaks will also be formed (one containing L-D plus D-L; the other L-L and D-D) but the label ratio in each peak will remain constant. Therefore, even when a wide variety of substances may be present in the sample, the ratio recording of the chromatograph output will be influenced only by optically active species. Overlapping peaks will interfere only insofar as they attenuate the shift in ratio by diluting the difference in label.

This approach therefore requires the fewest assumptions about the specific molecules being sought; naturally, there are technical considerations on the choice of a variety of reagents and columns best suited for different classes of substances.

Tritium and C^{14} were mentioned as differential labels only for purposes of illustration, though they might well serve for certain purposes. With halogenated reagents, only one radioactive isotope may be needed, electron capture and other methods giving excellent detection of total material. Other ultrasensitive techniques, such as neutron capture methods, alpha-particle backscattering, and so on, also suggest themselves. The greatest utility might be found from mass-spectrometric detection, e.g., with O^{18} labelling of the trifluoracetyl group and thermal cracking of the chromatograph effluent; with careful choice of materials, monitoring the m/m+2 ratio would give a very fast, highly sensitive recording for optical activity; the same instrument without cracking could give the full mass spectrum of just the interesting fraction, i.e., data from which to deduce the chemical nature of the optically active species.

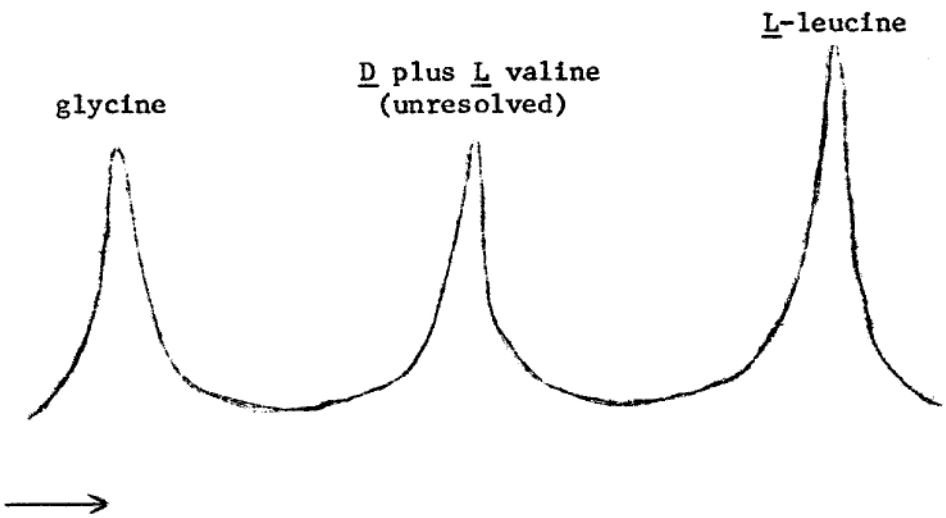
The work already done could be incorporated directly into a useful life-detecting experiment, namely for the properties of amino acids partly separated by another sub-system. We propose to continue our investigations on refinements along the lines indicated that would yield a system giving a general approach to the detection and identification of minute amounts of optically active materials.

Samples

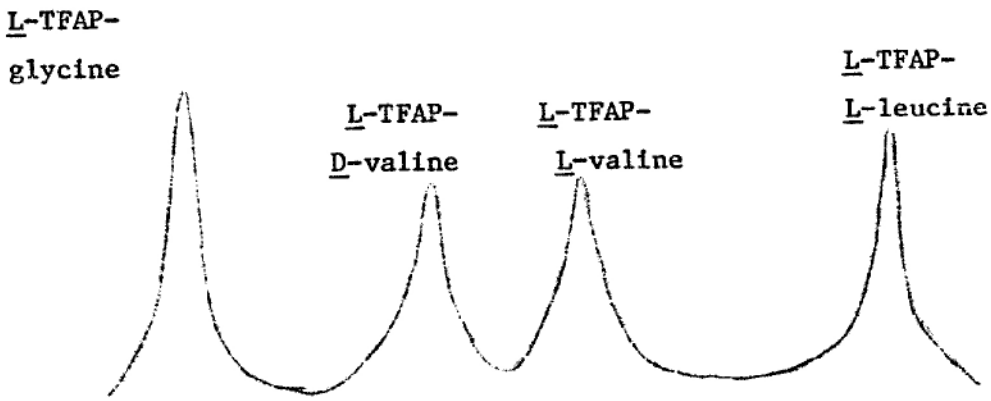
Our present thinking encompasses the utility of soil samples of the order of 1 to 10 grams, the collection of which would involve a subsystem commensurate with the complexity of the analyzer (i.e., gas chromatograph, mass spectrometer and thereon). The reagents and their handling should be manageable within a kilogram and a power requirement very small compared to the analyzers. There should be no problems of sterilization of these reagents. If means of scaling down these analyzers are found, samples of the order of 1 - 10 mg might plausibly be expected to yield an interesting result.

Collection of volatiles from the atmosphere also deserves consideration, perhaps with the help of morning dewfalls. It should be pointed out that on the permafrost model, most of the "volatile" material of Mars will be distilled or leached out and fossilized at some depth beneath the surface in equatorial regions. Subarctic zones roughly at the times of waves of darkening have the best chance of surface exposure, but digging at the equator should give a similar result - as would shaded crevices. Microorganisms might be expected to be most abundant between the surface and the permafrost and especially in the vicinity of such crevices.

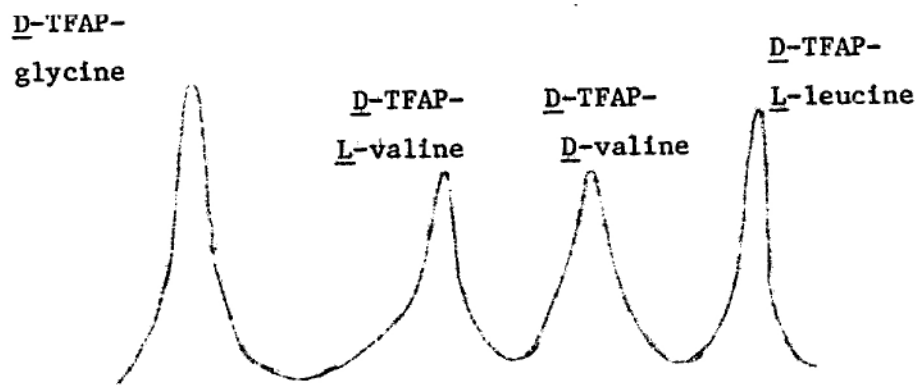
Consider a mixture of glycine (symmetric), DL-valine (racemic) and L-leucine (optically active) in equal amounts of each isomer. The following chromatograms would be realized. (TFA stands for trifluoracetyl; TFAP stands for trifluoracetyl-prolyl.)



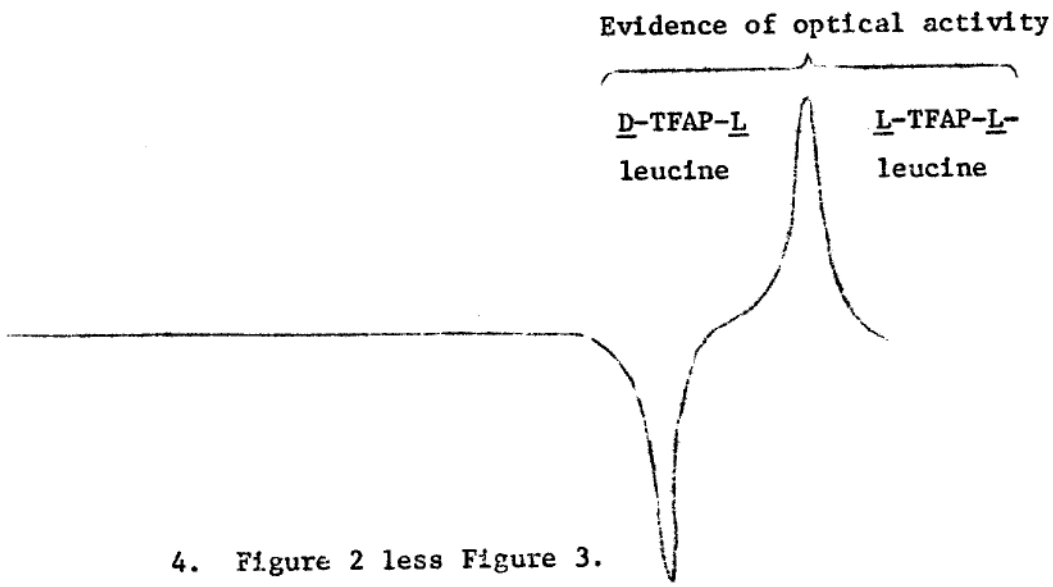
1. N-TFA amino acids



2. L-TFAP peptides



3. D-TFAP peptides



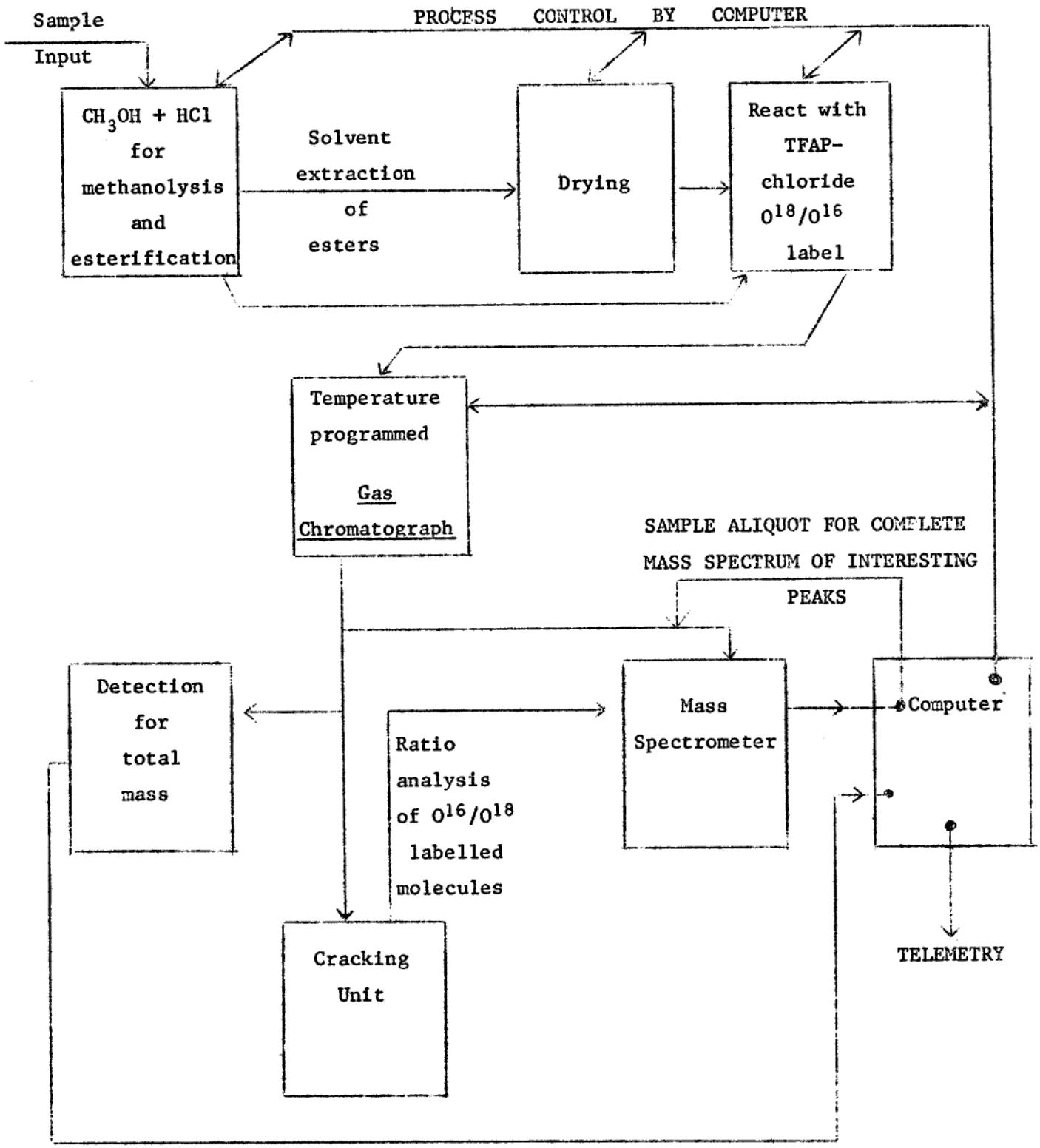
4. Figure 2 less Figure 3.

Figure 4 could be obtained either by (b) running parallel columns precisely calibrated and controlled with respect to the reaction with TFAP or (c) differential labelling of the TFAP used in a single reaction.

FLOW DIAGRAM

A Complete Subsystem for the Automated Biological Laboratory.

This would have the most flexibility, sensitivity and richness of information.



THE PASTEUR PROBE--a general assay for molecular assymetry*

The application of gas-liquid chromatography (g.l.c) to the detection of optically active molecules has been outlined in the preceding pages as a program closely analogous to our own thinking and experimentation. We propose some extensions and differences in emphasis. If our collaborative program is approved, we would expect to integrate our views into the final detail of the experiment, taking advantage of further preliminary work in both sets of laboratories, and more detailed specification of the capsule environment.

1. Not restricted to amino acids. The logical basis of the experiment is the role of molecular assymetry in defining the conformation of any polymer. We need make no fixed assumptions about amino acids (as such) as the methods will generally be applicable to any target molecules with free -OH or $-NH_2$ groups. As was pointed out, preliminary hydrolysis of the sample is likely to increase the yield considerably.

2. Choice of several reagents. While several likely candidates are already known, work should be continued to look for more promising reagents. We have noted some advantages and disadvantages for trifluoroacetyl-aminoacyl chlorides (especially proline); a-chloroalkanoyl chlorides; and trifluoroacetyl-thiazolidine carboxyl chloride. The use of these reagents differs only in detail from the process steps already summarized. Some simplification may be possible with further work. However, even as it stands, the repertoire will accomplish the tasks demanded.

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*Presentation from Professor J. Lederberg, as coinvestigator, representing the Stanford exobiology group. The supporting data are embodied in a manuscript "Optical Resolution of D,L Amino Acids by Gas Chromatography and Mass Spectrometry" by B. Halpern, J. W. Westley, Ingilt von Wredenhagen and J. Lederberg.

3. Ratio-detection with labelled reagents. When the components of the target material are undefined, asymmetric molecules can still be diagnosed by contrasting the chromatograms obtained first with one, then the other of a pair of enantiomeric couplers. Only an asymmetric species can generate a product, i.e., a diastereoisomer, which differs between the two runs. This two-step experiment can be executed with conventional reagents and detectors, but it places stringent demands on the homogeneity of the samples and the quantitative reproducibility of the entire process.

We have therefore sought ways of labelling the enantiomers, then mixing the D- and L- couplers before condensing them with the target material. With a detector that discriminates between the labels, the ratio will fluctuate sharply first in one direction, then the other, as the diastereoisomers from coupling with an asymmetric species are resolved within a single chromatogram. The resolution between the diastereoisomers, or among the various chemical species, need not be perfect to give a decisive result, nor will we necessarily be able to identify what the species is merely from the relative mobility on the g.l.c.

4. Application of the mass spectrometer (m.s.). One way of accomplishing this experiment has been realized. The labelled reagent was dideuterated L-(N-trifluoracetylthiazolidine-4-oyl chloride). In contrast to the undeuterated, D- coupler, peptides of the L-coupler produce an abundant fragment at m = 186 instead of m = 184. That is, the abundance ratio at these mass numbers bespeaks the resolution of the couplers. With the materials so far examined, the background at these mass numbers has been negligible; this would of course have to be determined by a control run.

Resolution at these mass numbers requires a rather capable m.s., though not necessarily beyond the scope of the present mission. We are therefore investigating other reagents that can be expected to allow a similar analysis

to be feasible in a lower mass range, overlapping the requirements of atmospheric composition analysis. For example, if the g.l.c. peaks are burned or cracked before being admitted to the m.s., hydrogen and deuterium can be analyzed directly, and the values compared with other readouts on the same peaks. The details of this system will be designed on the basis of further information on the capsule system so as to share as far as possible the use of the detectors available. A wide choice of these might be useful here. No fundamentally new art is required, although a great deal of detailed calibration is called for.

5. An inoculation experiment. The experiment so far consists of an observation of the existing statistics of asymmetric molecules at or near the Martian surface. It would be directly applicable to hydrolysates of soil samples or to condensates from the atmosphere.

If a drill is provided, the experiment can be extended by inoculating the soil with substantial quantities of a variety of racemic compounds. After varying periods of time, small samples of the surrounding soil would be retrieved and extracted, then analyzed for the relative abundance of the inoculated species. So far, this is a typical metabolism experiment; however, we now examine the optical specificity of the recovered material. Almost without exception, biological metabolic systems show substantial selectivity in the degradation of the metabolites (and appearance of new products). Knowing which materials have been degraded will also tell us a good deal about the orientation of the metabolic system. A large number of DL-compounds could be used together, the main limitation being background noise and possible mutual toxicity.

This approach is not claimed to be ultra-sensitive, but compensates by interfacing directly with the Martian crust. Furthermore, the material introduced will not everywhere be in large excess, answering a plausible argument against swamping a small sample used in a metabolic experiment with any prespecified environmental factor, even, for example, water.

Only a few preliminary experiments have been done to verify this experiment. However, in a crude test run with probably too much material, a detectable increase in the ratio of D:L alanine was seen in 24 hours after DL-alanine was presented to a soil that had been stored dry in the laboratory for several months.

6. Implementation of the proposed experiments. We give first preference to a close collaboration with the Ames group, which would have primary responsibility for engineering development, and ultimate responsibility for critical decisions affecting the experimental package. On this basis, the specifications for payload weight and power fall within the statements of the overall proposal. One advantage of this arrangement, from our standpoint, is that it would permit our laboratory to continue to focus its development efforts on systems for an Automated Biological Laboratory.

Whatever mechanism is adopted for managing the ~~laboratory~~ experimental package, we would seek an association with other users of g.l.c. and m.s. rather than attempt an independent effort that would preempt a large fraction of the available payload for a single approach.

7. Significance of this insertion. The opportunity to identify this part of the presentation helps to illustrate the relationship already established between the principal and co-investigators in a area where further work with a reasonable balance of independent scientific and collaborative development effort is clearly desirable. It should not be thought that we disassociate ourselves from the general proposal, for we have had many profitable consultations on it. However, the leadership and responsibility rest with the principal, and we expect Ames to take the initiative in expanding its collaboration to other academic coinvestigators.